Investigating Taste Function in the Ageing Population

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Abbreviations

The abbreviations used in this thesis are:

ADP adenosine diphosphate

ANOVA Analysis of Variance

ATP adenosine triphosphate

BCA bicinchoninic acid

BDNF brain derived neurotrophic factor

BP base pairs

BSA bovine serum albumin

CALHM1 calcium homeostasis modulator 1

CAMP Cyclic adenosine monophosphate

Cas CRISPR-associated

CAVI carbonic anhydrase VI

CD36 cluster of differentiation 36

CMC carboxylmethyl cellulose

CNG cyclic nucleotide gated

CNS central nervous system

CRISPR clustered regularly interspaced short palindromic repeat

CV circumvallate

CT chorda tympani

DAG diacylglycerol

DDM differential dynamic microscopy

DRK delayed rectifying K+ channels

DTT dithiothreitol

EMG electrogustometry

ENaC epithelium sodium channels

ER extensional rheology

FFA free fatty acids

fMRI functional magnetic resonance imaging

GABA gamma-Aminobutyric acid

GLAST glutamate-aspartate transporter

gLMS generalised labelled magnitude scale

GLP-1 glucagon like peptide-1

HEK293 human embryonic kidney 293 cells

HPLC High Performance Liquid Chromatography

HPMC hydroxypropylmethyl cellulose

HRP Horseradish Peroxidase

IP_3 inositol triphosphate

IP_3R3 ionositol triphosphate receptor 3

kDa kilodaltons

KO knock-out

LCFA long chain fatty acids

MCFA medium chain fatty acids

mGluR Metabotropic glutamate receptors

mPa. s millipascal-second

MSG monosodium glutamate

MUC mucin

OR olfactory receptor

P2X purinergic receptor

PAS periodic acid-Schiff

PBS Phosphate Buffered Saline

PCR polymerase chain reaction

PKD polycystic kidney disease channel

PG propylene glycol

PGM porcine gastric mucin

PIP Prolactin-Induced Protein

PIP_2 phosphatidylinositol biphosphate

PKA protein kinase A

PLCβ2 phospholipase C beta 2

PROP propylthiouracil

PRP proline rich protein

PTC phenylthiocarbamide

PUFA poly unsaturated fatty acids

qPCR quantitative polymerase chain reaction

RT-PCR reverse transcription polymerase chain reaction

SA sodium alginate

SAG salivary agglutinin

SDS-PAGE Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

SEM standard error of the mean

SNP single nucleotide polymorphism

SOC store operated channels

SS Sjögren's Syndrome

STIM 1 stromal interaction molecule 1

TAS2R taste receptor 2

TB taste bud

TBS tris buffered saline

TBS-T 1% Tween With TBS

TG triglycerides

TR taste receptor

TRC taste receptor cell

TRPM5 transient receptor potential melastatin 5

TRP transient receptor potential channel

VAS Visual analogue scale

VEG von Ebner’s glands

WMS whole mouth saliva

UWMS un-stimulated whole mouth saliva

SWMS stimulated whole mouth saliva

WHO World Health Organization

XG xanthan gum

5-HT serotonin

Abstract

The diet of the ageing population is known to deteriorate which is in part due to loss of taste sensation. Lack of interest in food leads to nutritional deficiencies, dehydration and reduced quality of life. The maintenance of taste function is dependent on the presence and function of saliva. Saliva bathes the taste buds and coats the oral mucosa to enable processing of foods and to provide a trophic stimulus for taste bud function. In the ageing population salivary flows tend to decrease for several reasons including disease, reduced water intake and the side-effects of some medications. This project firstly established the connection between quantity or alteration in quality of saliva and loss of taste sensitivity by comparing taste perception and physicochemical properties of saliva in older (60-90 years) and younger (18-30 years) groups. Saliva samples were collected in response to taste stimulation and rheological properties, viscoelasticity and viscosity, were assessed as well as composition particularly of proteins including mucins, cystatin S and carbonic anhydrase VI (CAVI). The second part of this study was to develop *in vitro* cellular models to investigate saliva-mediated modulation of specific taste receptor responses. Transfected TR146 cells over-expressing the TAS2R38 receptor and the SCC090 cell line that endogenously expresses TAS2R10 were used to measure intracellular calcium responses to bitter taste compounds. Models were tested using a fluorophore and confocal microscopy as well as a florescent plate reader. Saliva samples collected during the volunteer study were used to create a salivary layer over a confluent epithelial cell monolayer and the effects of saliva from older and younger groups were compared with regard to tastant diffusion and receptor activation. Reduced calcium responses to bitter taste compounds were observed when saliva from older adults was added to the confluent cells compared to saliva from younger adults. Responses to bitter tastants *in vivo* could be correlated to the calcium response in the *in vitro* model in the presence of saliva from younger subjects but not with saliva from older subjects. Levels of certain salivary proteins including mucins and the viscoelasticity of saliva samples correlated with taste receptor activation in the cell models and as such, the effect of physical properties of saliva on taste function was demonstrated *in vitro*.

1 Introduction

1.1 Mechanisms of taste

1.1.1 Anatomy and Innervation of the Oral Cavity

The oral cavity forms the beginning of the digestive tract, facilitating the formation of food bolus and initiating digestion through secretion of saliva (Figure 1-1 (German and Palmer 2006)). There are 2 main components of the oral cavity, the oral vestibule and the oral cavity proper (Chen and Engelen 2012). The vestibule is anteriorly limited by the upper and lower lips and laterally by the cheeks (Unger 1985). The internal boundary between the vestibule and oral cavity is formed by the teeth and gums (Unger 1985). The oral cavity itself is limited posteriorly by the oropharynx (where the pharynx connects to the oral cavity), superiorly by the palate and inferiorly by the floor of the mouth (German and Palmer 2006). The teeth perform the important role of chewing, shearing and shredding food, while masticatory muscles allow the jaw to move which facilitates the movement required for chewing (Koussoulakou, Margaritis and Koussoulakos 2009). The mouth is covered by a mucous membrane made up of stratified squamous epithelial cells (Squier and Kremer 2001).

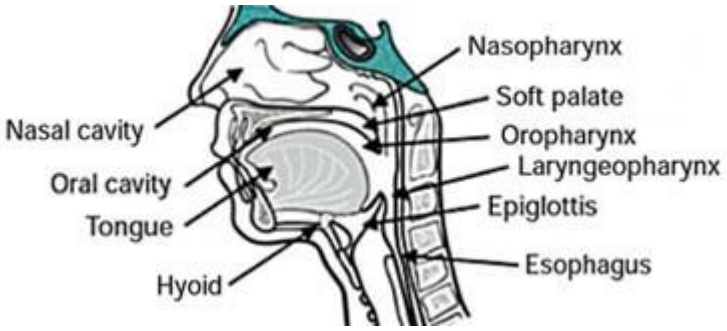


Figure 1-1 (German and Palmer 2006) Anatomy of the Oral Cavity

Salivary glands, including the major (parotid, submandibular and sublingual) and minor salivary glands, produce saliva needed to moisten and bind chewed food into a bolus which can be swallowed (Pedersen 2002, Denny, Ball and Redman 1997). The parotid gland is found anterior to the ear and Sternocleidomastoid muscle – a superficial muscle running from the clavicle and sternum to the mastoid process of the temporal skull bone, in the retromandibular fossa (Bialek et al. 2006). The submandibular gland is

located in the rear part of the submandibular triangle, limited by the digastric muscle and the mandible (Bialek et al. 2006). The sublingual gland can be found between the muscles on the floor of the mouth, adjacent to the mandible (Bialek et al. 2006).

The tongue is also part of the oral cavity with the circumvallate papillae marking the base (Chen and Engelen 2012). It is made up of four muscles, the genioglossus, hyoglossus, styloglossus and palatoglossus with intrinsic fibres the vertical, transverse and longitudinal (German and Palmer 2006). Motor innervation is supplied to the tongue by the lingual, glossopharyngeal and laryngeal nerves (Sawczuk and Mosier 2001). The nerve supply for taste sensation originates from the chorda tympani, glossopharyngeal and internal laryngeal nerves (German and Palmer 2006).

All of the superficial facial muscles described are innervated by branches of the facial nerve (VII) (German and Palmer 2006). The chewing muscles, masseter, temporalis and medial and lateral pterygoid muscles, act together to move the mandible for mastication (Chen and Engelen 2012, Hannam and McMillan 1994). The motor root of the trigeminal nerve provides innervation to these muscles (Hannam and McMillan 1994). The suprahyoid muscles, made up of the digastric, mylohyoid and geniohyoid muscles form the oral floor (Pearson, Langmore and Zumwalt 2011). Generally, they allow the jaw to recede and facilitate opening of the mouth, with the digastric muscle being the main muscle involved in jaw opening and the geniohyoid facilitating raising of the hyoid bone (Pearson et al. 2011, Van Eijden, Korfage and Brugman 1997). The soft palate is made up of several muscles, the tensor veli palantini, levator veli palantini, palato pharyngeus, uvulus and palatoglossus, which form a sheet like muscle involved in opening and closing of the airway simultaneous to swallowing (Matsuo and Palmer 2009). Branches of the trigeminal nerve provide general sensation to the oral cavity (Rodella et al. 2012). The maxillary nerve gives sensation to the upper oral cavity such as the teeth and palate while the mandibular nerve supplies the lower oral cavity such as the mucosa of the oral floor (Rodella et al. 2012).

1.1.2 Morphology, Location in Oral Cavity and Cellular Composition of Taste Buds

The human oral cavity is home to between 2000-5000 taste buds (Chaudhari and Roper 2010). Taste buds are located all over the oral cavity, mainly focussed on the tongue, pharynx, palate, epiglottis and the larynx. The taste buds located on the anterior two

thirds of the tongue are compacted into fungiform papillae, while those found on the anterior third are structured as circumvallate and foliate papillae (Sugimoto and Iseki 1994). Each human fungiform papillae holds anything from zero to 25 individual taste buds (Arvidson 1979). Each onion-like bud (Figure 1-2 (Bissell and Steele 2011)) is made up of around 100 cells which cluster together tightly, interwoven with nerve fibres (Finger 2005).

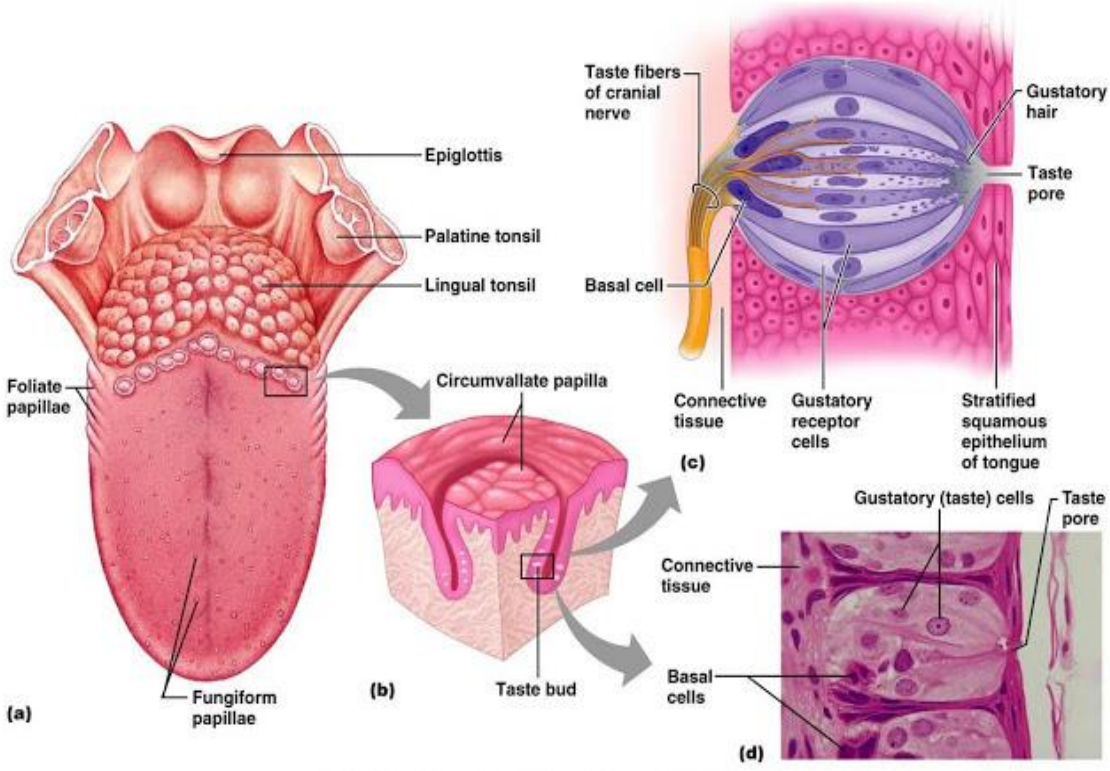


Figure 1-2 (Bissell and Steele, 2011) Histology of the Human Taste Bud

The cells making up taste buds can be classified morphologically into 4 types of cell, each with an important role in taste function (Roper 2013). Type I cells make up approximately half of all taste bud cells. They surround the other cells in the bud and are responsible for expression of certain molecules able to activate neuro transmitters involved in the taste sensation (Pumplin, Yu and Smith 1997).

Type ii cells make up a quarter of cells found in taste buds and are often referred to as "taste receptor cells" because they have specific receptor sites able to bind sugars (sweet taste), glutamates (umami taste) and bitter taste molecules (Ozdener et al. 2011). Type ii cells also express a number of proteins important in taste transduction including G proteins, α-gustducin and phospholipase c-beta 2 (PLC-β2) (Chandrashekar et al. 2006).

Type iii cells are thought to be responsible for the sour taste response and these cells make up 15% of all cells found in the taste bud (Ozdener et al. 2011). These "pre-synaptic" cells secrete several neurotransmitters to facilitate cell-cell signalling, vital for functionality of the taste system. When type ii cells release ATP, as a neurotransmitter resulting from taste excitation, they signal to type iii cells which respond via release of serotonin and norepinephrine (Huang et al. 2008). These two hormones act to create a negative feedback loop, whereby, type ii receptors are switched off to reduce conflicting messages being sent to the brain from taste bud signals, about more than one taste.

Finally, type iv cells (basal cells) are the cells from which other cell types are derived. They are the stem cells which eventually differentiate to become one of the other three types of taste cell described above (Sullivan, Borecki and Oleskevich 2010). Type IV cells reside in the base of the buds and allow for constant regeneration and renewal of taste cells, with a cell turnover of 5-10 days in adults (Okubo, Clark and Hogan 2009).

1.1.3 Taste Receptors and Transduction of the 5 Basic Tastes

Sweet Taste

Type ii cells have several different receptors on their surface which bind taste molecules. There are specific receptors for each tastant. Sweet tastes, such as sugar molecules activate T1R2 and T1R3 receptors (Figure 1-3 (Lee and Cohen 2015)). These T1R's are all G-protein-coupled receptors (GPCRs) (Chandrashekar et al. 2006). These receptors localize within the taste pore and combine in dimers (can be hetero or homologous) to form receptor complexes with sensitivity to sugar/sweet molecules (Hoon et al. 1999). Studies in mice, with knockout of either the T1R2 or T1R3 coding genes, have demonstrated huge reduction in taste responses to sugar and artificial sweeteners. Knockout of both receptors completely eradicated the ability of the mice to taste sweet substances, indicating a synergistic action of the heterologous receptor complex T1R2/T1R3 required for complete sweet taste function (Zhao et al. 2003a).

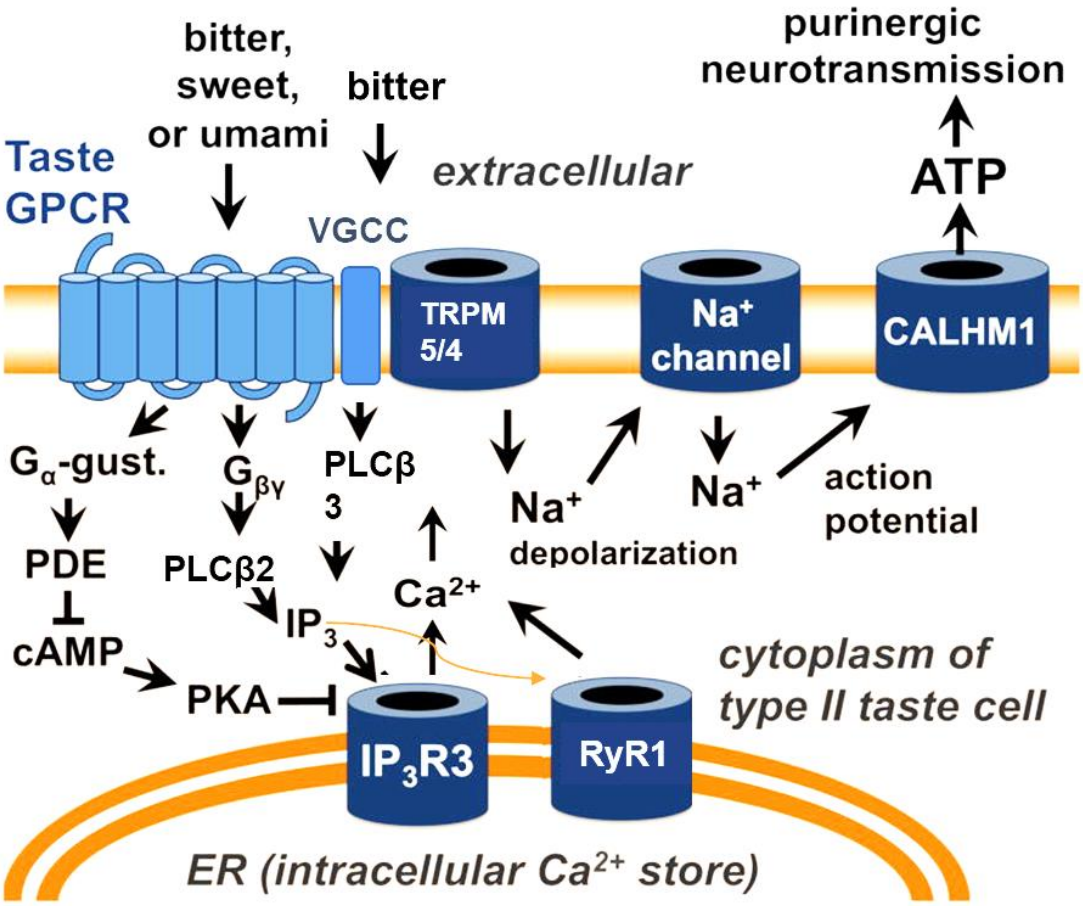


Figure 1-3 (Adapted from Lee and Cohen, 2015) Taste Transduction Pathway of Sweet, Bitter and Umami Tastants Mediated by G Protein/Phospholipase C Signalling Cascade

Interaction of sweet molecules with T1 receptors activates G α gustducin, and other G-protein α subunits (α14, α15, αq) or Gβγ subunits, which in turn interact with phospholipase C beta 2 (PLCβ2 – as shown in Figure 1-3) (Huang et al. 1999). This action stimulates the production of inositol triphosphate (IP_3) which opens IP_3R3 ion channels, causing release of calcium and a depolarization of the cell membrane. This reaction activates transient receptor potential melastatin 5 (TRPM5), which in turn stimulates a release of ATP, that is picked up by purinergic receptors on afferent nerves (Figure 1-3 (Lee and Cohen 2015)) (Huang et al. 2011, Huang and Roper 2010). A signal is then sent via the CNS, to the brain, causing a taste response (Kovacic and Somanathan 2012).

PLCβ2 knock-out mice display reduced but not complete loss of sweet, umami and bitter taste qualities (Dotson, Roper and Spector 2005). In addition, these mice remain responsive to high concentrations of bitter stimuli (Dotson et al. 2005). This suggests a PLCβ2 independent mechanism for taste sensation, particularly for aversive concentrations of bitter stimuli. A proportion of bitter sensing taste cells express voltage gated calcium channels (VGCCs), which may be involved in calcium signalling in response to bitter tastants (Hacker et al. 2008). VGCC signals evoked by bitter tastants may be mediated by PLCβ 3/IP3R1, since expression of these has been shown in the sub-set of taste cells which express VGCCs (Hacker et al. 2008). In addition, in mouse taste cells, expression of ryanodine receptor type 1 (RyR1) has been shown and may be involved in calcium release from intracellular stores following taste stimulation (Rebello and Medler 2010). RyR1 can be activated by calcium induced calcium release and may be activated following calcium release via IP_3R3 (Morales-Tlalpan, Arellano and Diaz-Munoz 2005). The dual action of RyR1 and IP_3R3 may be required in order to create and maintain a sufficient taste-evoked calcium response, to facilitate opening of TRPM5 channels and depolarisation of the cell required for neurotransmitter release (Rebello and Medler 2010).

Several studies have shown decreased taste responses in TRPM5 knockout mice (Ohkuri et al. 2009, Damak et al. 2006). However, only one study has found complete loss of behavioural and nerve responses to sweet, umami and bitter tastants following deletion of the TRPM5 gene (Zhang et al. 2003). This indicates the presence of a TRPM5 independent mechanism for sweet, umami and bitter taste sensation. Recently, TRPM4, a functionally similar voltage sensitive, monovalent cation selective channel,

was shown to be expressed in type ii and a sub-set of type iii taste cells and may be vital for transduction of tastants following the PLCβ2 signalling pathway (Liu et al. 2011, Banik et al. 2018). In single TRPM4 or TRPM5 knockout mice, there are residual Na+ responses to taste in type ii taste cells (Banik et al. 2018). However, double knockout of TRPM4 and TRPM5 in mice, led to complete loss of type ii taste cell responses to bitter, sweet and umami compounds, as well as absence of behavioural taste responses *in vivo* (Banik et al. 2018). Na+ responses in taste cells of TRPM5 KO mice are slower, and require higher stimulus concentration compared to that of TRPM4 KO mice Therefore, the initial intracellular Ca^2+ response to taste stimuli may be sufficient to activate TRPM5 and then, as the Ca^2+ levels rise, TRPM4 is also activated (Banik et al. 2018). The joint activation of both channels contributes to taste evoked Na+ influx, and the depolarisation of the cell is larger than when only one of these channels are activated. Since double KO of the two channels abolishes taste responses, this larger depolarisation is required to activate ATP release from the voltage gated Calhm1 channel (Banik et al. 2018). It was also demonstrated that the TRPM5-/- mice, used by Zhang et al. (2003), had reduced expression of IP_3R3 and TRPM4 in the CV papillae. Therefore the TRPM5 mutation was not neutral, which explains the total loss of taste function in these mice, since IP_3R3 is an important component of the upstream taste signalling pathway (Banik et al. 2018).

1.1.4 Umami Taste

Umami tastants are detected in much the same way as sweet (described above - Figure 1-3). The main T1 receptors involved with umami taste detection are T1R1 and T1R3, and type ii cells expressing these receptors bind umami taste molecules, such as amino acids/glutamates (Zhao et al. 2003a). Receptor complexes made up of T1R1/T1R3 are strongly activated by amino acids, specifically glutamate and αL-aspartate in humans, and this action may be greatly enhanced by purine nucleotides such as inosine monophosphate (IMP) (Nelson et al. 2002). The heterologous complex of T1R1/T1R3 receptors is required for umami taste, as T1R1 or T1R3 knockout mice display significantly reduced response to IMP enhanced amino acids (Zhao et al. 2003a). Knockout of both receptors results in significant loss of umami taste ability (Zhao et al. 2003a).

As described above for sweet tastant molecules, the umami taste follows a TRPM5 mediated pathway. The G proteins activated by the T1R1/T1R3 modality are Gβ3 and Gγ13 (Zhang et al. 2003).

Glutamate also has the capacity to depolarise taste cells, at concentrations lower than those required for gustatory detection, indicating the existence of multiple glutamate receptors in taste buds (Lin and Kinnamon 1999). Additionally, while the Zhao study described complete loss of umami taste in T1R1 or T1R3 knockout mice (Zhao et al. 2003a), other studies have shown that residual umami taste is retained in these knockout models (Damak et al. 2003, Kusuhara et al. 2013, Delay et al. 2006). Also, in isolated taste cells from these knockout animals, responses to glutamate and nucleotides (IMP) were retained (Choudhuri, Delay and Delay 2015). It should be noted however, that responses of isolated taste cells may not exactly parallel *in vivo* responses, as stimuli are not restricted to the apical membrane.

Metabotropic glutamate receptors, mGluR4 and the truncated form taste-mGluR4, are expressed in taste buds. L-2-amino-4-phosphonobutryic acid, an mGluR4 agonist, has been shown to cause similar behavioural responses to those induced by monosodium glutamate, in rats and mice (Delay et al. 2000). Also, mGluR4 knockout mice display reduced nerve responses to umami stimuli (Yasumatsu et al. 2015). mGluR1 and its truncated form, taste-mGluR1, are also expressed in taste buds. Antagonists of mGluR1 and taste-mGluR1 cause reduced chorda tympani and glossopharyngeal nerve responses to glutamate (Yasumatsu et al. 2015). Also, glutamate may be released, by afferent nerves, to modulate taste responses. Indeed, afferent taste nerves express vesicular glutamate transporters, VGLUT 1 and 2 (Vandenbeuch et al. 2010, Braud, Boucher and Zerari-Mailly 2010).

Ionotropic glutamate receptors and kainate receptors are expressed in taste cells, suggesting that taste cells can be activated by glutamate, as a neurotransmitter (Caicedo, Kim and Roper 2000b, Caicedo, Jafri and Roper 2000a, Kim, Caicedo and Roper 2001, Vandenbeuch et al. 2010). These are likely to be type iii cells, due to responses to glutamate in these cells at typical neurotransmitter concentrations, while type ii cells mainly respond at taste level concentrations (Vandenbeuch et al. 2010). Neurotransmitter-like concentrations of glutamate, can evoke intracellular calcium release in the basal processes of taste cells, where the synapses between taste cells and nerve fibres are found (Vandenbeuch et al. 2010, Caicedo et al. 2000a). Similarly low

concentrations of glutamate can also lead to serotonin release from type iii cells, which acts as a negative feedback loop to decrease ATP release from type ii cells following taste stimulation (Huang, Grant and Roper 2012). If glutamate is indeed a neurotransmitter in the taste bud, a mechanism for reuptake would be required. As such, type I cells have been shown to express a glutamate-aspartate transporter (GLAST) providing further evidence for a taste signal modulating role of glutamate (Lawton et al. 2000).

1.1.5 Bitter Taste

The transduction of bitter tastants follows the same pathway as umami and sweet tastants (Figure 1-3), however different type ii receptor complexes are involved (Zhang et al. 2003). T2R/TRB complexes are responsible for bitter taste transduction, with approximately 20% of all type ii cells expressing these receptors (Ruiz-Avila et al. 2001). Rat and mouse models have shown that only cells which express gustducin, can detect bitter tastants (Ruiz-Avila et al. 2001). There are around 30 different T2R receptors, expressed in human type ii cells, and different combinations of receptors respond to one or several of the wide variety of bitter chemicals consumed in the diet (Matsunami, Montmayeur and Buck 2000). Previously it was shown that almost all T2Rs are co-expressed in type ii cells so that taste cells would not discriminate between different bitter compounds, but merely detect differences in intensity of bitterness (Mueller et al. 2005). However, more recent evidence points to heterogeneity of TAS2R expression in bitter taste cells, with limited tuning of individual cells toward subsets of bitter tastants (Behrens et al. 2007).

1.1.6 Sour Taste

Sour tastants have a very different taste mechanism and transduction pathway to umami, sweet and bitter tastants. Until recently, it was believed that acid molecules were detected via entry of H^+ through membrane channels in type iii cells (Huang et al. 2006, Chandrashekar et al. 2006). Several types of 2-pore domain potassium leak conductance channels (K2P family) are expressed in taste cells and have been shown to respond to intracellular acidification (Richter et al. 2004). Another theory involved PKD (Polycystic Kidney Disease) receptors, namely PKD1L3 and PKD2L1. PKD's are a family of TRP receptors expressed in type iii taste cells. PKD1L3/PKD2L1 subunits are co-expressed to form a Ca^{2+} permeable channel (Ishimaru and Matsunami 2009, Kataoka et al. 2008). The receptors appear to respond to very low pH with an "off

response". Therefore, the gated channel only opens following removal of the acidic stimulant (Inada et al. 2008). This may be relevant *in vivo*, as strong acidity evokes a salivary response which may dilute the acid sufficiently to open these channels and elicit a sour taste response (Inada et al. 2008). Some studies have shown that PKD2L1 knockout mice are completely unable to respond to acid tastants (Huang et al. 2006). However, other studies in mice lacking the PKD2L1 gene have demonstrated residual sour taste responses (Horio et al. 2011). Mice lacking the PKD1L3 gene, or with targeted mutation, may have normal acid taste sensitivity, which suggests that these channels are not responsible for acid taste sensing (Horio et al. 2011, Nelson et al. 2010). In addition, HEK293 cells transfected with PKD1L3/PKD2L1 receptors do not respond to acid stimuli (Chang, Waters and Liman 2010). As such, the role for this hetero-dimer, in sour taste, is uncertain.

Since earlier studies investigating the role of PKD1L3/PKD2L1 used very strong acidification to elicit a response, these channels may be involved in acid taste response from strong acids. Weak acids illicit a stronger response in the gustatory nerve than strong acids - at the same pH, which points to the involvement of intracellular acidification as a second step in sour taste transduction. This is because weaker acids can diffuse across the cell membrane, while strong acids cannot (Lyall et al. 2001). The discovery of a Zn^{2+} sensitive proton channel, expressed in sour tasting cells, has shed light on this (Chang et al. 2010). This channel allows protons to be shuttled across the cell membrane during acid stimulation, causing intracellular acidification. This acidification is sufficient *in vitro* to induce action potentials due to the charge carried by H+ ions (Chang et al. 2010).

However, this is likely an initial step in the sour taste transduction cascade and may work synergistically with cell depolarisation, caused by blocking of K^+ channels. Cell depolarisation can be caused by influx of Na^+ or Ca^{2+} or by blocking of outward K^+ flow (Ye et al. 2016). In sour taste cells intracellular acidification has shown to block K^+ current. Inward-rectifier potassium channels ($K_{ir}2.1$) are the most likely K^+ channel involved in this mechanism. Tissue specific ablation of $K_{ir}2.1$ significantly reduced the magnitude of resting K^+ current, showing that this channel is important in maintaining resting K^+ balance in sour taste cells (Ye et al. 2016). Non-sour taste cells also express $K_{ir}2.1$ channels, and in greater density than sour taste cells. They do not respond to intracellular acidification in the same way, because the greater number of $K_{ir}2.1$

channels on the cell surface creates a higher magnitude of K^+ current. This means that a much greater level of acidification would be required to block the channels and cause depolarisation (Ye et al. 2016). The small magnitude of K^+ current in type iii taste cells allow weak acids to stimulate depolarisation of the cell, resulting in the firing of action potentials which cause neurotransmitter release and a sour taste response (Ye et al. 2016). Furthermore, type iii cells display Zn^{2+} sensitive proton influx, in response to reduced cytosolic pH, which blocks the $K_{ir}2.1$ channel (Chang et al. 2010).

1.1.7 Salt Taste

There are 2 distinct components of salt taste transduction in mammals, comprising the amiloride sensitive (AS) and insensitive pathways (AI). The AS pathway is thought to mediate salt flavour perception or saltiness and is specific to sodium. The AI pathway is not specific to a single salt and is involved in aversion to high salt concentrations, activated to prevent excessive salt consumption, which can be harmful in the body (Chandrashekar et al. 2010). Amiloride sensitive salt taste is believed to be transduced via direct entry of Na^+ through channels in the receptor cell membrane.

These channels are epithelial Na channels (ENaCs), which can be blocked by amiloride, a diuretic drug. The channels allow for a stationary inward current of Na^+ ions from the lumen, across the apical cell membrane into the cell, causing cell depolarisation (Heck, Mierson and DeSimone 1984). ENaC subunits are expressed on the fungiform taste buds, while significantly less expression is evident in vallate taste buds (Kretz et al. 1999). This is of interest, as amiloride insensitive salt taste is most prominent on the posterior tongue where the vallate taste buds reside (Lin et al. 1999). Amiloride sensitive salt taste is most prominent on the anterior tongue, where fungiform taste buds reside (Lin et al. 1999). The AI salt taste response has been characterised in two taste cell types, type iii sour sensing cells and type ii bitter receptor cells (Oka et al. 2013).

TRPV1 channels have also been linked with AI salt taste, however this is controversial since TRPV1 knockout mice exhibit a fairly normal response to aversive salt taste (Ruiz et al. 2006). AI salt response is inversely associated with anion size of the salt, that is, larger anions produce a smaller response. This is likely due to the impermeable nature of large anions, through taste pore tight junctions (Ye, Heck and DeSimone 1991). There are two proposed populations of type iii cell involved with AI salt transduction (Lewandowski et al. 2016). The first is slightly dependent on anion size and has higher

osmotic response. The second is highly dependent on anion size and has reduced osmotic response (Lewandowski et al. 2016).

1.1.8 Fat Taste

Recently, there has been much interest in the fat taste, and the mechanisms behind a response elicited by consuming fatty/creamy foods. Mice and rats show preference for long chain fatty acids (LCFA), over triglycerides (TG), while humans can detect LCFA, medium chain fatty acids (MCFA) and short chain fatty acids (SCFA) (Takeda, Imaizumi and Fushiki 2000, Tsuruta et al. 1999, Mattes 2009). Breakdown of TG to free fatty acids (FFA), in the oral cavity, relies on salivary enzymatic breakdown (Kawai and Fushiki 2003). In rodents, a form of lipase is secreted by the von Ebner salivary glands (VEG) and lipase activity is also present in human saliva (Hamosh and Scow 1973, Voigt et al. 2014, Pepino et al. 2012). There is a high inter individual variation in salivary lipase concentrations in rodents, which may explain the high inter-individual variation in sensitivity to LCFA (Mattes 2009). Delayed rectifying K^+ (DRK) channels may play a role in fat taste reception (Gilbertson et al. 1997). Polyunsaturated fatty acids (PUFA) have been shown to inhibit DRK channels, eliciting taste bud cell depolarisation required to initiate neuro transmitter release, due to an accumulation of positive charges (Gilbertson et al. 1997). This likely plays a modulatory role, in taste. For example, in the presence of PUFA, concentrations of saccharine below usual detection levels, are preferred to saccharine alone, in rats (Gilbertson et al. 2005).

Cluster of differentiation 36 (CD36) is a candidate fat taste receptor (Pepino et al. 2012). CD36 is expressed in the membrane of taste bud cells, as well as in other cell types, and binds to saturated and unsaturated LCFA (Baillie, Coburn and Abumrad 1996). Deletion of the CD36 gene in mice results in reduced preference for LCFA. However, post-ingestive signals can override the effects of this deletion. Oil trained CD36 knockout (KO) mice have normal attractive behaviour for fats (Sclafani, Ackroff and Abumrad 2007). This suggests that oral and post-ingestive signals combine, to create an attraction for fat *in vivo*. Humans with a single nucleotide polymorphism (SNP), which reduces CD36 expression, have an increased threshold for LCFA and a greater acceptance of fat taste than those with the wild-type CD36 gene (Pepino et al. 2012).

G protein coupled receptors (GPCR), GPR40 and GPR120, may also have roles in fat taste detection (Cartoni et al. 2010). GRR40/120 KO mice have been shown to have reduced responses to oral fat stimulation (Cartoni et al. 2010, Ancel et al. 2015, Galindo et al. 2011). However, GRP40 has not been found in the taste buds of mice or humans, whereas GPR120 is expressed in abundance in the taste bud and non-gustatory oral epithelium in both (Cartoni et al. 2010, Ancel et al. 2015, Galindo et al. 2011). None the less, activation of GPR120 by LCFA alone is not sufficient to generate a sensation in mice or humans (Godinot et al. 2013). Little is known about the subsequent signalling cascade following stimulation by LCFA. It may be similar to that of sweet, umami and bitter tastants, with activation of PLCβ_2 and IP_3 production, triggering release of Ca^{2+} from endoplasmic reticulum (ER) stores (El-Yassimi et al. 2008). This depletion of ER stores may result in activation of stromal interaction molecule 1 (STIM 1) - an ER calcium sensor, which then opens store operated channels (SOC) to allow an influx of Ca^{2+}, leading to cell depolarisation (Dramane et al. 2012). STIM1 KO mice have a complete loss of fat preference (Dramane et al. 2012). TRPM5 -/- mice also have abolished preference for fat, suggesting a role in Ca^{2+} entry in taste bud cells following ER store depletion, after LCFA stimulation of CD36 (Liu et al. 2011). Nerves involved in fat taste signalling are similar to those involved in basic taste reception (Cartoni et al. 2010). The chorda tympani and glossopharyngeal nerves are involved in transfer of signal following fat taste stimulation, as denervation of both has been shown to cause loss of fatty acid preference in mice (Gaillard et al. 2008).

1.1.9 Neurotransmitters and Taste Transduction

Type iii cells are the only taste cells which have synaptic structures, including synaptic vesicles, and expressing enzymes required for biosynthesis of neurotransmitters (Chaudhari and Roper 2010). Type ii cells have no neuron like features, however they are still capable of transmitting taste information to neurons, via ATP release (Chaudhari and Roper 2010). Type ii cells also release acetylcholine which activates M3 muscarinic receptors, expressed on adjacent taste cells, to potentiate ATP release following taste stimulation (Dando and Roper 2012, Ogura 2002). Type iii cells release several neurotransmitters in response to taste, including serotonin (5-HT), GABA and norepinephrine (Meredith, Corcoran and Roper 2015, Cao et al. 2009, Dvoryanchikov et al. 2011). GABA has an inhibitory effect on type ii cell ATP release, as these cells

express $GABA^a$ and $GABA^b$ receptors, indicating a negative feedback role (Dvoryanchikov et al. 2011, Cao et al. 2009).

How type ii cells release ATP upon gustatory excitation is not entirely clear, however recent studies have shown the potential role of Calcium Homeostasis Modulator 1 channels (CALHM1) (Taruno et al. 2013). CALHM 1 is a plasma membrane ion channel, which is voltage gated and activated by membrane depolarisation or reduction of extracellular calcium (Ma et al. 2012). CALHM1 KO mice display almost complete loss of sweet, bitter and umami taste response, while retaining sour and salt taste (Taruno et al. 2013). Decreased extracellular calcium, or high K+ induced depolarisation, cause ATP release from CALHM1 transfected cells, but not from mock transfected cells. CALHM1 is expressed in type ii taste cells, and may have a role in ATP release upon membrane depolarisation, caused by the taste response (Taruno et al. 2013). Pannexin-1 is another candidate ATP release channel expressed in type ii taste cells (Huang et al. 2007). Pannexins are gap junction hemi-channels and in some cells also act as transmembrane channels, which allow transfer of small molecules, including ATP, between the cell and extracellular space (Bao, Locovei and Dahl 2004). Taste stimulated ATP release from type ii cells is inhibited by carbenoxolone, a specific pannexin-1 inhibitor (Huang et al. 2007). However, in contrast to CALHM1 KO mice that exhibit severely impaired taste ability, pannexin-1 KO mice have normal ATP release from type ii cells and display normal behavioural and neural responses to taste (Romanov et al. 2012, Tordoff et al. 2015, Vandenbeuch, Anderson and Kinnamon 2015). Pannexin-1 may therefore function as an additional ATP release channel in taste receptor cells, or play a role in cell-cell communication between taste cells (Romanov et al. 2012, Tordoff et al. 2015, Vandenbeuch et al. 2015).

Serotonin (5-HT), released by type iii cells, may have multiple roles in taste. Firstly, 5-HT release from type iii cells can activate adjacent type ii cells, which express the $5\text{-}HT_1{}^a$ receptor - an inhibitory G-Protein coupled receptor (Huang, Dando and Roper 2009). This causes an inhibition of type ii cells, acting as a negative feedback loop to stop conflicting messages being sent to the brain from taste bud signals about concurrent tastes (Jaber et al. 2014). Also, this negative feedback allows for adaptation of the taste buds, so that the neuronal signals from type ii cell receptors are decreased following prolonged exposure to a tastant (Chaudhari and Roper 2010). Secondly, 5-HT release upon sour and possibly also salt taste stimulation, may directly activate $5\text{-}HT_3$

receptors on afferent nerves (Larson et al. 2015). Despite this, it has been shown that ATP release is crucial for transmission of all taste responses. Therefore, 5-HT must work alongside ATP, to excite gustatory nerves upon taste stimulation (Vandenbeuch et al. 2013).

Purinergic receptors (P2X's), expressed by sensory afferent nerve fibres, are activated by ATP binding and are required for taste responses via gustatory nerves (Finger et al. 2005). P2X2/P2X3 heteromers are the most likely receptor complex for detecting ATP in gustatory nerves, since immunostaining of nerve fibres in the fungiform papillae of rodents has shown that P2X2 almost always co-localises with P2X3 (Huang et al. 2011). Furthermore, P2X2/P2X3 double knockout mice have a completely abolished taste response to all 5 basic tastes (Ishida et al. 2009, Huang et al. 2011). Single knockouts of either receptor had minor effects on taste phenotype, therefore, both are capable of forming functional homomeric receptors in gustatory nerves (Finger et al. 2005). Additionally, taste receptor cells from these mice secrete significantly reduced levels of ATP in response to taste stimulation (Huang et al. 2011). P2X2 receptors are also expressed by type ii cells, suggesting presence of an autocrine positive feedback loop in which released ATP activates P2X2 on type ii cells, to stimulate further release (Huang et al. 2011). Type ii cells also express P2Y1 receptors, which can be activated by ADP, a breakdown product of ATP (Huang et al. 2009). Type 1 cells express NTPDiphosphohydrolase2, an ectoATPase, in the cell membrane. ATP released upon gustatory stimulation can be broken down, by NTPDiphosphohydrolase2, into ADP which can activate P2Y1 receptors for further ATP release and a greater taste response (Bartel et al. 2006) (Huang et al. 2009).

Expression of several peptide transmitters has been demonstrated in taste cells. These include cholecystokinin, vasoactive intestinal peptide and neuropeptide Y (Zhao et al. 2005, Herness et al. 2002, Shen et al. 2005). GLP-1 (glucagon like peptide-1) is expressed specifically in some sweet sensing αgustducin/T1R3 type ii cells and 25% of type iii cells (Shin et al. 2008, Martin et al. 2009). GLP-1 receptors are also found on taste bud afferent nerves. GLP-1R knockout mice display reduced neural and behavioural responses to sweet stimuli, suggesting local GLP-1 signalling, occurring to potentiate the sweet taste response (Shin et al. 2008, Martin et al. 2009, Takai et al. 2015). Initial GLP-1RR-/- mouse studies found a modulatory effect of GLP-1 expressed by type iii cells on sour taste with hypersensitivity in knockout mice (Shin et al. 2008).

However, more recently it was shown that GLP-1RR KO mice had no difference in behavioural or neural response to sour stimulus, compared to wild type mice (Takai et al. 2015, Shin et al. 2008).

1.1.10 Innervation of the Taste Buds and Salivary Glands

The tongue, taste buds and taste bud cells are closely connected to a vital network of nerves. This allows neurotransmitter signals to be transmitted from the taste buds to the CNS, for identification and detection of taste compounds. The taste receptor cells do not have a unique nerve supply, but are closely linked to the facial sensory nerve fibre endings (Kovacic and Somanathan 2012). The anterior tongue is innervated by the Chorda Tympani, which originates from the geniculate ganglion, and taste buds making up the fungiform papilla are closely connected to the nerve endings of this large facial nerve (nerve VII) (Yarmolinsky, Zuker and Ryba 2009). The taste buds found in the circumvallate papillae have neuronal supply from the Glossopharyngeal nerve (nerve IX), while taste buds embedded in the foliate papillae are innervated by branches of both the Chordia Tympani and the Glossopharyngeal nerves (Roper 2013).

Exactly how gustatory afferent nerve fibres encode for different taste modalities remains controversial. There are two possible theories. Firstly, the labelled line model in which nerve fibres are tuned to specific taste qualities (Chaudhari and Roper 2010). Secondly, the combination model in which nerve fibres are broadly tuned and decode individual taste information according to activity patterns across several fibres (Chaudhari and Roper 2010). A recent study used calcium imaging of mouse geniculate ganglion neurons, following taste stimulation, to identify dedicated neurons for each of the basic tastes (Barretto et al. 2015). This suggests that each different taste quality is coded for by separate ganglion neurons, which are selective for each of the five taste qualities. It was also shown that some nerve fibres are “multi-tuned” and can respond to taste mixtures or combinations of more than one taste. However, the numbers of such neurons may be small, compared to single taste tuned neurons. Approximately three quarters of all neurons tested were tuned to only one taste quality. The remaining twenty five percent were composed of multi-tuned neurons, half of which were tuned specifically to bitter-sour stimulation. This is likely because of the dual sensitivity of some bitter taste cells to low pH.

Gustatory afferent nerve fibres, which express 5-HT_3^a receptors, preferentially form contacts with type iii cells. This suggested that they may provide a specific line of taste information transmission for sour taste sensing and, since salt taste may also be transduced by type iii cells, also for salt taste (Stratford et al. 2017). Previous studies have shown that single tuning of gustatory nerve fibres is dependent on concentration of tastant. For example at low concentration nearly all neurons respond to a single taste quality, while at higher concentrations the majority become more broadly tuned, and respond to multiple taste qualities (Tomchik et al. 2007). This may be similar to the combinational tuning seen in olfactory nerve responses, to varying concentrations of odorants (see 1.2, Olfaction).

1.1.11 Taste Thresholds

The taste detection threshold is usually described as the concentration of a basic tastant required to elicit a response, from the taster. Different tastants have different threshold concentrations. For instance, average thresholds have been reported to be 0.0165M for sucrose (sweet taste), 0.0316 M for NaCl (salt taste), 0.000743 M for tartaric acid (sour taste) and 0.0000203 M for quinine hydrochloride (bitter taste) (Yamauchi et al. 2002). Aside from inter-taste variances, taste threshold values are also subject to great individual variation. As such, individuals have a unique palate and ability to detect basic taste compounds (McMahon, Shikata and Breslin 2001).

Age, medication, anxiety, mood and disease states are all factors affecting taste thresholds. Certain medications are known to reduce taste function, including anti-depressants, beta blockers, diuretics, aspirin and diabetes drugs (Doty, Shah and Bromley 2008).

1.1.12 TRP Receptors

Aside from the basic 5 tastes, there are other compounds found in the diet which can elicit a response in the oral cavity. Heat, pungency, cooling, astringency and fattiness are all sensations felt on the tongue but the chemicals responsible are not considered "tastes".

Compounds such as capsaicin, the chemical responsible for heat in chillies, can cause a stinging/burning sensation in the mouth. This is due to the direct activation of TRPV1 channels, expressed by epithelial cells found on the tongue and all over the oral mucosa (Marincsák et al. 2009, Wang et al. 2011). Pain, heat, protons and vanilloid agonists can all activate TRPV1 receptors (Caterina et al. 1997, Tominaga et al. 1998). Capsaicin binds to one of several TRPV1 vanilloid pockets, causing influx of calcium and other ions, which create a net inward current and membrane depolarization (Boillat, Alijevic and Kellenberger 2014, Elokely et al. 2016). This can generate firing of action potentials and ATP release, which may activate P2X receptors on surrounding nerve fibres (Park et al. 2003, Elokely et al. 2016, Wang et al. 2011, Mandadi et al. 2009). TRPV1 receptors are also expressed by nerve fibres surrounding the taste buds. Therefore, direct action of capsaicin on sensory afferents, originating in the chorda tympani and trigeminal nerves, also contribute to its chemo-sensation (Ishida et al. 2002, Katsura et al. 2006). Prolonged exposure of TRPV1 channels to capsaicin results in desensitisation due to an accumulation of Ca^{2+} (Koplas, Rosenberg and Oxford 1997). Capsaicin induced TRP activation can also lead to increased thresholds for basic tastants (Simons, Boucher and Carstens 2003). Additionally, capsaicin and other TRP agonists, including menthol, have shown to activate bitter taste pathways and therefore may be considered taste compounds as well as trigeminal stimuli (Green and Hayes 2004, Green and Schullery 2003).

Menthol, icilins and camphor are all cooling agents, which create a cooling sensation in the mouth (Cliff and Green 1994, Green 1985). TRPM8 channels are expressed by trigeminal ganglia nerves in the tongue and possibly epithelial cells (Abe et al. 2005, Wang et al. 2011). It should be noted however, that the concentrations of TRP agonists used to show expression of TRP receptors in oral epithelial cells were much higher than that which would stimulate TRP receptors on nerve cells. This indicates that responses shown may be due to activation of other ion channels. In addition, the same study used ruthenium red as a TRP receptor blocker, to show that responses to TRP agonists were

specific. Ruthenium red is a general ion channel blocker therefore, reduced calcium response to TRP agonists in the presence of ruthenium red, may be due to blocking of other ion or calcium channels (Cibulsky and Sather 1999). As such, it is not entirely clear whether TRP receptors are indeed expressed in the oral epithelium and what role this would play in oral sensations in response to TRP agonists.

TRPM8 channels are sensitive to cold temperatures, as well as menthol and cooling agents, and upon their activation, an increase in intracellular calcium occurs, by influx of extracellular Ca^{2+} or by release from intracellular stores (Peier et al. 2002, Reid, Babes and Pluteanu 2002). This leads to membrane depolarisation and finally neurotransmitter release, possibly of glutamate, facilitating transmission of sensory information to the CNS (Peier et al. 2002, Tsuzuki et al. 2004, Behrendt et al. 2004). TRPM8 may be expressed on the endoplasmic reticulum (ER) membrane and as such, menthol and cold stimuli could also cause intracellular calcium release from ER stores, directly (Abeele et al. 2006, Zhang and Barritt 2004). Additionally, depletion of Ca^{2+} from stores, caused by TRPM8 activation, may lead to opening of store-operated channels (SOC) and influx of extracellular calcium (Abeele et al. 2006).

However, recently the role of TRPM8 in Ca^{2+} release from internal stores following menthol stimulation has come into question. HEK293 cells, which do not express TRPM8 channels endogenously, still show an increase in intracellular calcium in response to menthol stimulation (Mahieu et al. 2007). Additionally, the calcium response to menthol in these cells and in LNCaP cells, which do express TRPM8, was potentiated by increased temperature. This is in contrast to previous studies, which have shown that menthol induced TRPM8 channel Ca^{2+} release is increased by cold temperatures (Mahieu et al. 2007). This evidence points to the existence of a TRPM8 independent Ca^{2+} release system in response to menthol, the nature of which is still unclear. TRPM8 independent responses to menthol may be the mechanism behind irritation and burning sensations, elicited by high menthol concentrations (Cliff and Green 1994, Mahieu et al. 2007). Since low concentrations of menthol elicit a cooling sensation, these are likely caused by stimulation of TRPM8 channels (Behrendt et al. 2004, Mahieu et al. 2007).

1.1.13 Taste Perception and Saliva Secretory Response

It is well established that gustatory stimuli are one of several salivary stimuli, causing increased flow rates which are concentration dependent and tastant specific (Guinard, Zoumas-Morse and Walchak 1997, Neyraud et al. 2009, Ilangakoon 2011). Citric acid and other sour tastants are responsible for the greatest increases in saliva secretions (Hodson and Linden 2006). MSG or umami tastants produce the next greatest flow rates, followed by salt and then sweet and bitter taste which have similar effects on saliva secretion (Hodson and Linden 2006, Dawes and Watanabe 1987). Parotid gland salivary secretion has been used previously as a measure of gustatory responses (Chauncey and Shannon 1960). Some taste compounds do not exhibit a linear flow response to increasing concentrations, perhaps because those compounds recruit more than one taste response pathway(Chauncey and Shannon 1960).

Additionally, taste compounds likely stimulate different glands in different proportions, since the rheology of saliva has been shown to change, depending on taste compound (Vijay et al. 2015, Stokes and Davies 2007),. The labial minor salivary glands also exhibit a reflex response to all five basic tastes, which has been positively correlated with taste perceptions in healthy adults (Satoh-Kuriwada et al. 2018). The salivary reflex response is regulated by the parasympathetic nervous system. Increased labial blood flow has been shown, in response to taste stimulation, and could be positively correlated to taste perceptions and labial salivary flow (Satoh-Kuriwada et al. 2018). TRP agonists, such as capsaicin and menthol, also induce salivary secretion, above basal levels (Nasrawi and Pangborn 1990, Lawless 1984). The effect of TRP agonists on saliva secretion is also dependent on compound used. The rheology of saliva may be altered by different TRP agonists suggesting stimulation of the mucin producing glands and parotid gland in different proportions (Houghton et al. 2017).

Although saliva secretion is thought to be a reflex response to taste stimuli, the increase in flow rate is not always well correlated to the perceived intensity of the tastant (Guinard et al. 1997). The absence of correlation between the two may be because every individual has different experiences with tastes and food. Therefore, each person has a different reference point when taking part in sensory testing. Taste perception can be based on individual experience and are not always a definitive value for taste responses. As such, taste perception is subjective, while salivary reflex response may be an objective measure of taste response (Engelen et al. 2003). Twin studies have shed light

on the genetic vs environment impact on individual taste perceptions. For example, a study comparing salt and sour taste thresholds in monozygotic (identical) and dizygotic (fraternal) twins showed that fifty percent of the variation in sour taste can be accounted for by genetics (Wise et al. 2007). However salt taste recognition thresholds were shown to have no link with genetics but a strong link with the shared environment of the twin sets (Wise et al. 2007). Additionally, intensity perceptions of sweet taste solutions have been shown to be hardly inherited if at all. There was no within pair correlation in sets of monozygotic twins, indicating a strong environmental component for sweet taste perception (Keskitalo et al. 2007).

Another reason for the lack of correlation between reflex saliva and taste perception is that individuals have different saliva composition, therefore baseline adaptation between individuals will vary. Saliva contains Na^+ and K^+ among other ions, so, as the taste buds are constantly bathed in saliva they may adapt to the levels of salt in the oral fluid. An individual with high salivary salt levels might experience greater adaptation and have a reduced response to salt stimuli (Delwiche and O'Mahony 1996, Matsuo et al. 1994, Matsuo and Yamamoto 1992, McBurney and Pfaffmann 1963, Contreras and Catalanotto 1980). Rat neural and behavioural responses to salt were shown to be significantly lower after the tongue was bathed in saliva. In the case of neural responses to salt taste, this could be directly correlated to the concentration of electrolytes (Na^+, K^+, Cl^-, and $HCO3^-$) present in the saliva (Matsuo and Yamamoto 1992, Matsuo et al. 1994). In humans, it has been shown that raised salivary Na^+ concentrations - induced by chewing, leads to increased taste thresholds for salt (Delwiche and O'Mahony 1996). Additionally, adaptation of the tongue with sodium chloride solutions was shown to lead to reduced intensity of subsequent salt taste solutions (Bartoshuk, McBurney and Pfaffmann 1964)

However, the methods used in such studies can induce bias. For example, use of chewing to induce increased Na^+ in saliva would by default increase flow rate. This might cause reduced perception of salt taste due to a dilution effect. Indeed, differences in individual salivary flow rates may impact upon sensitivity to tastants. It has been shown that an increased salivary flow rate can cause reduced sensitivity to salt and sour tastants, indicating a buffering and diluting effect of saliva on tastants (Heinzerling et al. 2011). However, a different study found that subjects with a low stimulated salivary flow rate perceived sour and bitter stimulus as less intense than high stimulated flow

rate subjects (Lugaz et al. 2005). It could be that those with reduced sensitivity to taste stimulus also have a reduced reflex salivary response or a reduced salivary flow rate could be responsible for reduced taste response as tastants are transduced to the taste pore less effectively in those individuals (Lugaz et al. 2005). Subjects with a low stimulated flow rate did perceive sour/bitter tastants for significantly longer than high flow rate subjects indicating efficient clearance of tastants and buffering capacity of saliva (Lugaz et al. 2005).

1.2 Olfaction

Olfaction, or the sense of smell, begins within the mucous membrane of the nasal cavity. There are 3 main cell types involved in olfactory sensation - bipolar primary olfactory neurons, supporting cells and basal or stem cells, from which new olfactory sensing cells are generated (Moulton and Beidler 1967). A central pole of the sensory cell body extends into an axon which joins with other axons and forms nerve bundles which project to the olfactory bulb (Buck and Axel 1991). The nerve bundles terminate within the glomeruli and form synapses with mitral cells (Graziadei and Graziadei 1986). Cilia extend from the dendritic processes of olfactory neurons, at the epithelial surface, and lay over the olfactory epithelium within a mucus network (Nakamura and Gold 1987, Getchell, Margolis and Getchell 1984).

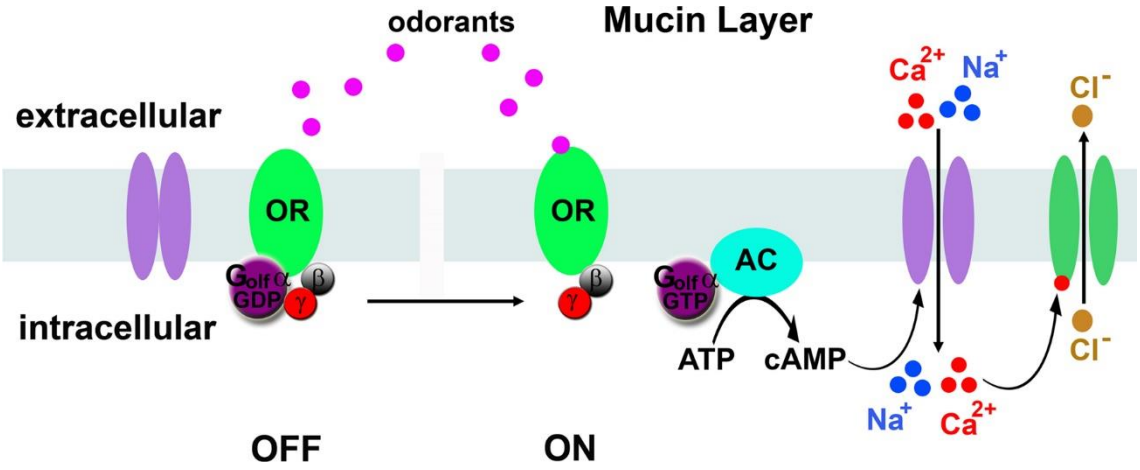


Figure 1-4 (Ha 2009) Schematic diagram depicting the cAMP-mediated transduction pathway of olfactory-receptor neurons

Olfactory sensory cells have receptor proteins, within their membranes, which bind olfactory stimulant molecules leading to activation of the G protein, G_{olf} (Jones and Reed 1989). G protein activation activates adenylate cyclase-catalysed production of cyclic adenosine monophosphate (cAMP) (Figure 1-4 (Ha 2009)) (Breer, Boekhoff and

Tareilus 1990). cAMP is capable of changing the conformation of cyclic nucleotide gated (CNG) channels, in the cell membrane, and the opening of these channels allows influx of Na^+ and Ca^{2+} into the cell (Zufall, Firestein and Shepherd 1994). As a result, the negative charge of the cell is shifted, and action potentials are created which are transported along the olfactory cell axon, into the brain (Nakamura and Gold 1987).

Olfactory receptors are GPCR proteins, part of a family of over 1000 receptor types (Buck and Axel 1991). The odorant molecule binds to the receptor site on the GPCR which undergoes allosteric conformational changes upon ligand binding becoming activated (Kaupp 2010). Each olfactory receptor cell expresses only one type of receptor (Malnic et al. 1999). As such, olfactory receptor cells act in a combinational manner, in response to odorant mixtures (Malnic et al. 1999). Axons of olfactory neurons, which express the same receptor, converge within the olfactory bulb at specific areas, in just a few of the glomeruli. Therefore, each glomerulus is dedicated to only one type of smell (Ressler, Sullivan and Buck 1994). An odorant can be recognised by different combinations of olfactory receptors, depending on the concentration of the odour. This explains why some smells are identified differently at different concentrations (Malnic et al. 1999).

1.3 Saliva Composition and Secretion

1.3.1 Composition of Saliva

Saliva is a hypertonic, mucoserous fluid originating in the oral cavity with many components relevant to oral health, taste and general health (Almeida, P. 2008). Although a relatively weak solution, made up of 99% water (Humphrey, SP. *et al* 2001), the remaining small levels of salivary proteins, electrolytes and nitrogenous products are a powerful mixture, each vitally important to the healthy functioning of saliva.

1.3.2 Proteins

Functional proteins in saliva play a wide range of important roles within the oral cavity. This includes lubrication, protection of teeth and oral surfaces, anti-bacterial and immunologic functions, buffering capacity and enzymatic activity (Bradley, Beidler and Doty 2003). Whole mouth saliva contains, on average, 1.5-2mg protein per ml - a relatively low protein concentration compared to other biological fluids, such as blood plasma (Streckfus and Guajardo-Edwards 2011). Total salivary protein has been attributed to over two-hundred different proteins and peptide complexes (Bradley et al. 2003), with more recent studies using proteomic methods describing over 3000 different proteins present in whole mouth saliva (Amado, Ferreira and Vitorino 2013). each with specific biochemical properties, important for function and health.

Mucins are a family of high molecular weight glycoproteins, which are responsible for much of the rheological properties of saliva. Mucins can hold large amounts of water and impart viscoelasticity to saliva to maintain lubrication of mucosal surfaces (Humphrey and Williamson 2001). There are three main forms of mucin found in the oral cavity, MUC 1, MUC5 and MUC7. Each type of oral mucin has different molecular weight and structural properties which give rise to different functions in the mouth (Amerongen, Bolscher and Veerman 1995, Nielsen et al. 1996, Pramanik et al. 2010). Mucins also play a part in protection against pathogens in the mouth, as they are able to aggregate bacterial/virus cells. Mucins can form complexes with other anti-microbial proteins. Mucin activity is concentrated on the surfaces of the oral cavity where mucins create a thick sticky barrier, forming a protective layer over the mucosa to impede pathogenic invasion (Wu, Csako and Herp 1994).

Immunoglobulins are another type of protein found in saliva (De Almeida et al. 2008). There are three main types of immune proteins in saliva, IgA, IgG and IgM. There is some secretory IgA (sIgA) in saliva although most is derived from plasma and is the most abundant salivary immunoglobulin (Lehner, Cardwell and Clarry 1967, Green 1954). These proteins work to prevent damage in the oral cavity by aggregating bacteria, neutralizing viruses and preventing attachment of bacteria cells to oral tissues (Humphrey and Williamson 2001).

Salivary enzymes can assist with digestion, anti-bacterial action and buffering of the oral cavity (Chauncey et al. 1954, Nakamura and Slots 1983). Amylase is a starch digesting enzyme which makes up almost half of the total salivary protein content (De Almeida et al. 2008). It mediates the initial stages of carbohydrate digestion, breaking down larger starch molecules into smaller sugars (Rosenblum, Irwin and Alpers 1988, Pedersen et al. 2002). Low levels of lipase activity have also been found in saliva from rats, with a minor role in fat digestion – initiating the break-down of dietary lipids, (Pritchard, Dawes and Philips 1967, Hamosh and Scow 1973).

Enzymes such as lysozyme, lactoferrin and peroxidase all contribute to protection of the oral cavity, against pathogens, and are part of the non-immunologic, anti-bacterial system in saliva (Tenovuo 2002). Lysozyme works by breaking down the bacterial cell wall, causing the cell contents to leach into surrounding fluid, inactivating the bacteria (Pollock et al. 1987). Lactoferrin is able to bind to free iron in the mouth and has an anti-microbial effect against pathogens which require iron to survive (Arnold et al. 1982, Nikawa et al. 1993). Peroxidase can catalyse an oxidation reaction of salivary thiocyanate, creating hypo-thiocyanate, which has strong anti-bacterial properties (Tenovuo and Pruitt 1984).

Low levels of hormones including stress response hormones, such as cortisol, steroids and thyroid hormones, are found in saliva. Measurement of such hormones in saliva can provide useful information about overall health of the individual, and is non-invasive (Vining, McGinley and McGinley 1986). Carbonic anhydrase VI, or gustin, is another important salivary hormone. It is a zinc binding protein, which may be involved in maintenance and growth of taste buds as well as enzymatic activity (Padiglia et al. 2010, Henkin 1978).

Proline rich proteins (PRPs) are also present in salivary fluid (Carpenter and Proctor 1999). They can be either acidic, basic or glycosylated and each type has a different function in the mouth (Bennick 1982). Acidic PRPs play a role in protection of teeth and oral surfaces, as they modulate surface binding activities of certain pathogenic bacteria (Newman et al. 1993). PRPs are also known to bind to tannins, with a protective effect against the astringent nature of such compounds (Gibbins 2013, Jöbstl et al. 2004). Astringency, or the puckering sensation elicited by certain polyphenol compounds, occurs as a result of reduced lubricity in the oral cavity because of precipitation of PRPs and salivary mucins (Jöbstl et al. 2004, Lee, Ismail and Vickers 2012, Gambuti et al. 2006). Polyphenols bind to salivary proteins to form aggregates, due to the formation of hydrogen bonds and hydrophobic interactions (Bennick 2002). Astringent compounds may also disrupt the salivary film and mucosal pellicle. This leads to reduced spreading of mucin, across the oral epithelium, which increases sensations of oral dryness (McColl et al. 2009, Nayak and Carpenter 2008).

Histatins are a small group of proteins in saliva which can protect against fungal infections, particularly from invasion by Candida fungi (Xu et al. 1991, Edgerton et al. 1998, Helmerhorst et al. 1999). They are also able to inhibit some bacterial enzymes and protect the mouth in this way (Gusman et al. 2001). Histatins 1, 3 and 5 have also been identified as proteins involved in the wound healing effects of saliva (Sun et al. 2009).

Statherin is a phospho-peptide which, like histatins, is involved in protection of the oral surfaces (Proctor et al. 2005, Harvey et al. 2011). Statherin may play an important role in the regulation of calcium levels in the mouth, for remineralisation of tooth enamel (Hay et al. 1984). It also has lubricating properties and binds to hydroxyapatite to initiate the formation of the protective surface layer of saliva found on teeth, known as the enamel pellicle (Li et al. 2004).

Proteins from the cystatin family are also involved in protection of tooth enamel via the protective pellicle by binding to hydroxyapatite and facilitating mineral absorption, into the tooth enamel (Siqueira et al. 2007). They are also involved in anti-bacterial protection in the oral cavity, as they can inhibit cysteine proteinases which contribute to periodontal disease (Baron, DeCarlo and Featherstone 1999, Ito et al. 2008, Ganeshnarayan et al. 2012)

Glycoprotein 340 (gp340), also known as salivary agglutinin (SAG), is another important glycoprotein with a high carbohydrate content (Rundegren 1986, Lee et al. 1989). It is strongly anti-viral and anti-bacterial and so aids in the protection against infectious agents in the mouth (Bikker et al. 2002, Malamud et al. 2011). Similarly to statherin and cystatins, SAG can bind to calcium and play a role in the protection of teeth, via the enamel pellicle (Carlen et al. 1998).

1.3.3 Electrolytes

Electrolytes are the main inorganic components of saliva, which add to the buffering, pH control, anti-microbial action and tooth remineralisation capacity of saliva (Almståhl and Wikström 2003). Bicarbonate is one of the main electrolytes found in saliva (Sand 1951). The presence of bicarbonate, in saliva, maintains a neutral pH to ensure that the oral environment is less suitable for pathogenic bacteria colonisation (Helm et al. 1982). A neutral pH also helps populate the mouth with beneficial bacteria, which may prefer this environment (Marsh 2009). Phosphates present in saliva have a similar role to bicarbonate, neutralising acids in the mouth to ensure the oral environment is suitable for beneficial bacteria, while also protecting against overgrowth of pathogens (Humphrey and Williamson 2001).

Sodium is another electrolyte found in saliva. It is important for taste function, having an adaptive effect on taste buds so that thresholds for salty tastes are elevated. This allows sweet taste compounds to be detected at a similar level to salt compounds and thus encouraging an energy rich diet (Delwiche and O'Mahony 1996). Past studies on mice and rats have demonstrated that removal of the adaptive saliva solution, which naturally bathes taste buds, leads to reduced taste threshold for salt (Matsuo et al. 1994). This results in a threshold for salt taste which is much lower than that of sweet taste. However, when the adaptive saliva was not removed, the thresholds for both sweet and salty were very similar (Matsuo and Yamamoto 1990). Sodium is also vital for encouraging flow of fluid from salivary glands to keep the oral cavity hydrated. This is because of the positive charge of sodium which, in conjunction with other salivary ions, creates a potential difference between lumen and acinar cells. This creates an osmotic gradient causing water to flow from the blood through the acinar cells and into the salivary ducts (Turner and Sugiya 2002).

Calcium is an electrolyte in saliva which is important for remineralisation of tooth enamel again via the protective pellicle covering teeth (Hay, Schluckebier and Moreno 1986).

1.3.4 Nitrogenous Products

Small amounts of urea have been found in saliva. Urea acts as a buffer, as it is metabolised by plaque creating ammonia to increase salivary pH (Dawes and Dibdin 2001, Stephan 1943). Urea also has an inhibitory effect against pathogens, reducing their ability to multiply and thereby assisting in the regulation of bacterial numbers in the oral cavity (Zabokova, Sotirovska and Ambarkova 2012).

1.3.5 Salivary Secretion

There are three major saliva glands in the oral cavity which facilitate salivary secretion. The three glands are termed the parotid, submandibular and sublingual glands. These major salivary glands are assisted by the minor salivary glands which contribute around ten percent of the total salivary flow (Proctor 2016).

The parotid gland supplies the mouth with approximately 20% of total un-stimulated saliva (Schneyer 1956, Holsinger and Bui 2007). It is a paired gland, located opposite the first molars (Holsinger and Bui 2007). This gland is composed of serous acinar cells, which secrete less mucous fluid and therefore the secretion is more watery and lower in viscosity than other saliva (Mese and Matsuo 2007). It is mainly involved in the production of stimulated saliva and is partly responsible for the increased salivary flow upon stimulation such as chewing (Shannon 1962, Sas and Dawes 1997). Parotid saliva contains high levels of amylase, PRPs and agglutinins with very small amounts of mucins - explaining the watery consistency and low viscoelasticity (Carpenter 2013).

The submandibular gland is found on the floor of the oral cavity and produces the greatest amounts of saliva of all the major glands, contributing around 60% of total salivary flow (Humphrey and Williamson 2001). It is composed of a complex of serous and mucous acinar cells and produces a viscous fluid, due to high mucin levels (Mese and Matsuo 2007, Tabak 1995).

The sublingual gland is also located on the floor of the mouth (Rosen and Bailey 2001). It contributes approximately 8% of total saliva production (Humphrey and Williamson 2001). The sublingual gland is made up of mucous acinar cells which produce a mucin

rich secretory fluid, with high surface rheology (Mese and Matsuo 2007). This gland also contributes relatively large amounts of lysozymes (Noble 2000, Stuchell and Mandel 1983).

The minor salivary glands include the palatinal and lingual, namely the von Ebner's glands, although there are hundreds of minor salivary glands in the oral cavity found all over the oral mucosa (Dawes and Wood 1973, Proctor 2016). Most of the minor glands are composed of mucous acinar cells, releasing a viscous fluid (Hand, Pathmanathan and Field 1999). von Ebner's glands are found in close association with circumvallate papillae and may play a role in taste bud function since the taste buds may be bathed in secretions from these glands (Gurkan and Bradley 1988).

Saliva secretion begins with stimulation of the parasympathetic and sympathetic nerves in the autonomic central nervous system (CNS). Stimulation of the parasympathetic nerve triggers a series of intracellular messages followed by calcium release and opening of Cl^- channels. Cl^- is secreted into the lumen creating a shift in electro-negativity and flow of sodium ions across tight junctions between acinar cells, as the cell attempts to create an electro-neutral environment (Mese and Matsuo 2007). The secretion of NaCl creates an osmotic gradient and water is drawn from the blood into the lumen (Turner and Sugiya 2002). At this stage the saliva is isotonic, however, the salivary ducts modify this by reabsorbing NaCl to create a hypotonic secretion (Melvin et al. 2005). Stimulation of the sympathetic nerve causes increased levels of cyclic monophosphate (cAMP) within cells which activates protein kinase A (PKA) (Baum and Wellner 1999). PKA leads to release of secretory granule contents, through exocytosis, contributing to the range of proteins found in saliva (Turner and Sugiya 2002, Takuma and Ichida 1994).

Unstimulated salivary flow rates are variable, depending on the study, but as a guide, a very low salivary flow may be reported as less than 0.1ml/minute, a low flow is between 0.1-0.2ml/minute and a normal flow rate is over 0.2ml/minute (Flink et al. 2008).

1.4 Rheology of Saliva

Saliva is a complex mixture of water, mucinous proteins and ionic compounds. It has an intricately balanced viscosity, with a strongly elastic nature and low surface tension. All these aspects can be considered the rheological properties of saliva and are important in

maintaining healthy salivary function. Taste, mouth feel, oral microbiological ecology and oral epithelium are all maintained by saliva and the rheology of saliva. Saliva can bind to oral surfaces and provide a protective film via viscoelastic mechanisms. Salivary viscosity must remain constant even at shear rates as high as 160^{s-1} (as occurs during speech) to ensure sufficient lubrication and protection of the oral mucosa (Waterman et al. 1988). Viscosity of saliva is directly related to the concentrations of proteins, mucins and glycoproteins and can be characterised as the dissipated energy during flow (Vissink et al. 1984). The multi layers of proteins in saliva result in high viscoelasticity, with a highly rigid layer found at the saliva-air interface. This layer contains high concentrations of statherin that forms a crystalline structure with calcium ions. Beneath this layer is another layer of aqueous saliva (Proctor et al. 2005).

Saliva from different glandular sources has different rheology due to the presence of varying amounts of mucins, glycoproteins, proline rich proteins and ions such as calcium and bicarbonate, which play a role in protein binding (Carpenter 2013). Saliva from submandibular/sublingual sources has a high mucin content of MUC5B and MUC7 which contribute to the gel forming and viscoelastic properties of saliva. Parotid saliva, described above, has a lower viscosity and almost no elasticity (Zussman, Yarin and Nagler 2007).

Further to this, saliva secreted during stimulation differs in its rheological properties, compared to unstimulated saliva. This is mainly due to the higher contribution of parotid saliva during stimulation. The type of stimulation is also an important factor affecting rheology of saliva. Stokes and Davies (2007) demonstrated that saliva stimulated by citric acid had a much higher viscoelasticity compared to mechanical (chewing) stimulated saliva despite both methods of stimulation evoking a similar flow rate. Different sources of stimulation likely evoke a salivary flow response from different glandular sources thus altering the compositional and physical properties of the secretion (Stokes and Davies 2007).

Age, disease state and medication can also affect the physical properties of saliva. Studies have shown that advanced age may have an effect on salivary flow and viscoelasticity, with older adults having a highly concentrated, viscoelastic saliva with a much lower (0.17ml/min) un stimulated flow rate than that of younger adults (0.45ml/min) (Zussman et al. 2007). In a recent meta-analysis of 47 studies looking at salivary flow rates and ageing, an average decrease of 0.168 mL/min was shown for

unstimulated whole salivary flow rate in older adults compared to younger. A decrease of 0.293 mL/min was shown for stimulated whole salivary flow rate. Submandibular/sublingual flow rates were reduced by 0.015 mL/min for unstimulated and 0.040 mL/min for stimulated on average (Affoo et al. 2015).

1.4.1 Extensional Rheology

The extensional rheology of saliva is an important property which is related to swallowing, speech, taste and lubricating functions in the oral cavity. Extensional rheology, or elasticity, can be considered as the amount of energy stored during flow (Waterman et al. 1988). The extensional properties of saliva (stringiness or spinnbarkeit (Figure 1-5 (Zussman et al. 2007)) are mainly due to the presence of mucins, which have highly glycosylated subunits that connect via disulphide bridges (Pearson, Allen and Hutton 2000). MUC5B forms dimers through disulphide bonding of the C-terminal domains of monomeric units (Figure 1-6a (Authimoolam and Dziubla 2016)). Additionally, interactions between the N-terminal domains facilitate the formation of oligomers (Kesimer et al. 2009). In this way, a mucinous gel network is created imparting elasticity and viscosity to saliva. Mucin gels are a tangled network and the amount of water contained within the network determines the visco-elastic properties (Verdugo et al. 1987). Mucin conformation, concentration and structure all affect the capability to form a gel. Age, medication and disease may cause changes in mucin, which subsequently increases feelings of dry mouth reported in individuals suffering from xerostomia (Zussman et al. 2007, Chaudhury et al. 2016). The extensional rheology of saliva is dependent on the glandular source (Van der Reijden, Veerman and Nieuw Amerongen 1993).

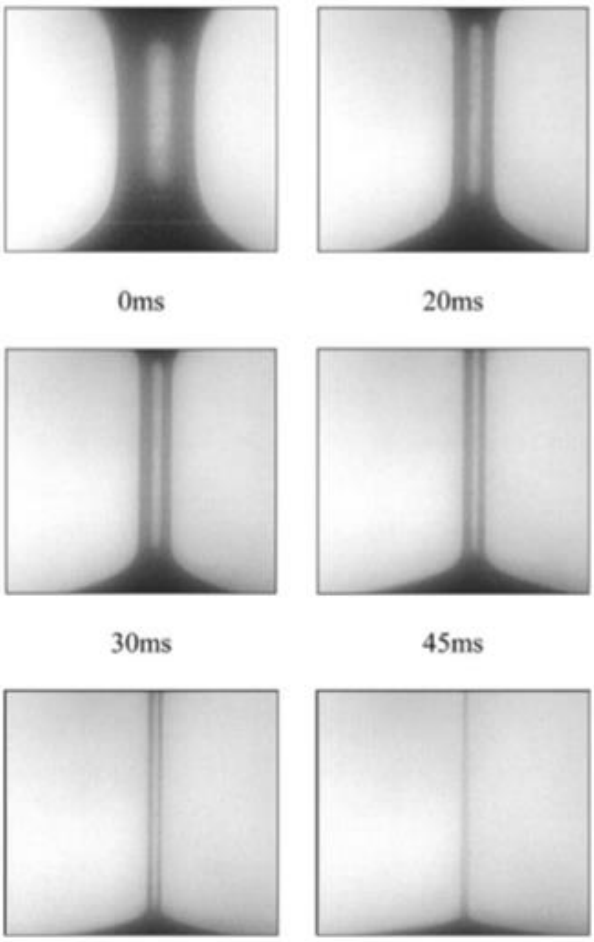


Figure 1-5 (Zussman et al. 2007) Elongational Flow of Parotid Saliva

Stimulated saliva has reduced viscoelasticity compared to un-stimulated, perhaps due to the increased proportion of parotid saliva following stimulation (Stokes and Davies 2007). However, minimising the contribution of parotid saliva in stimulated saliva collection still leads to reduced viscoelasticity, compared to un-stimulated submandibular/sublingual saliva. Therefore, reduced mucin concentration is not the only factor in the reduced viscoelasticity of stimulated saliva (Vijay et al. 2015). Chewing-stimulated saliva has increased bicarbonate levels, with an increased pH, compared to un-stimulated saliva (Vijay et al. 2015). Bicarbonate can chelate calcium ions, because of the strong attraction between calcium and carbonate which form calcium complexes, such as CaCO3 and CaHCO (Neuman et al. 1956). Mucin chains are polyionic and therefore the concentrations of ions within saliva affect swelling of the mucin network (Tam and Verdugo 1981). The highly charged carbohydrate rich domains of mucin facilitate binding to ions (Kesimer et al. 2009). Interchanging of Na^{+} ions with Ca^{2+} leads to contraction of the gel network since the carboxyl group of mucin sialic acid interacts with calcium ions to form a cross linked gel (Forstner and Forstner

1975). Therefore, sequestering of calcium ions alters the mucin chain conformation which leads to hydration of the gel and reduced viscoelasticity (Chen et al. 2010). Additionally, exchange of calcium ions for monovalent Na^+ causes an increase in osmotic pressure, because each calcium ion is replaced by two Na ions and thus the number of ions within the gel is increased (Kesimer et al. 2009). Due to the increased number of ions, water molecules enter the gel via osmosis. pH also plays a role in mucin gel conformation and therefore in salivary viscoelasticity. At acidic pH, hydrophobic regions of mucins are exposed because of protonation and subsequent cleavage of salt bridges which form between the negatively charged carboxylates and positively charged amino groups (Bansil and Turner 2006). Therefore, at low pH, hydrophobic interactions with adjacent mucin subunits are increased (Figure 1-6 b (Authimoolam and Dziubla 2016)) (Hong et al. 2005b).

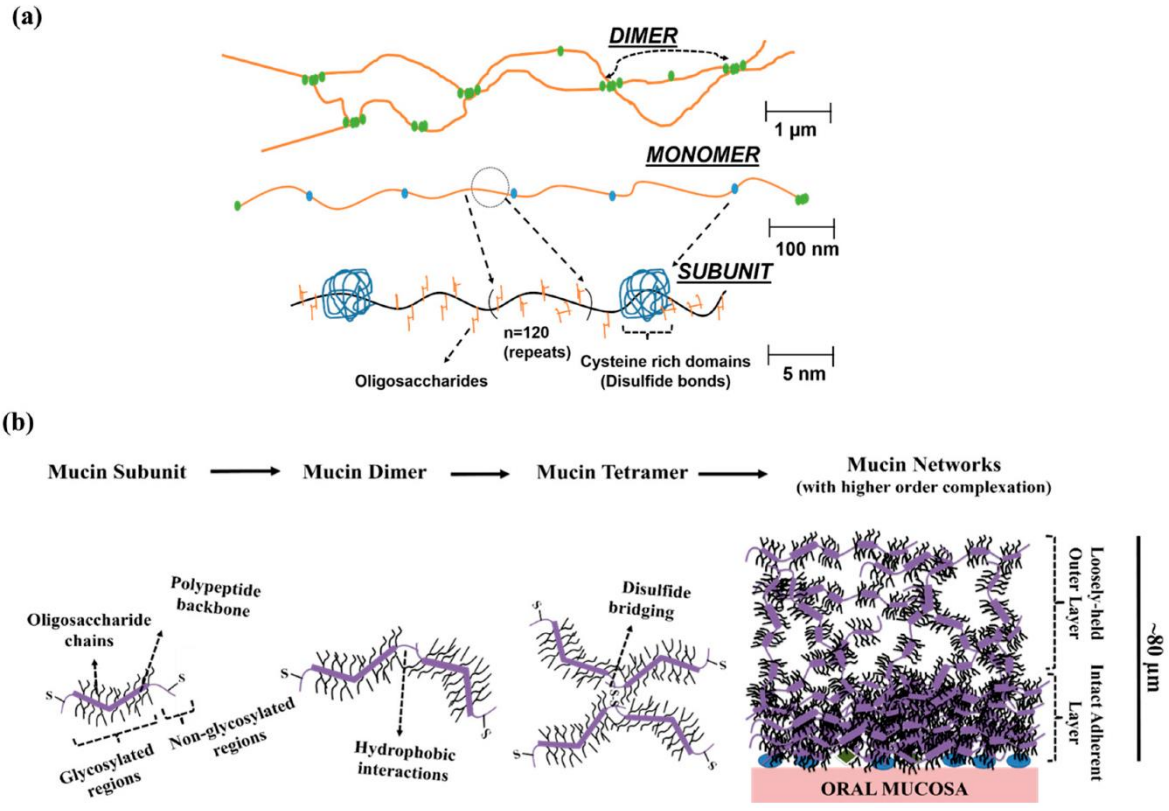


Figure 1-6 (Authimoolam and Dziubla 2016) Gel Network Formation of Mucin

(a) Sub-unit, monomer and dimer formation of mucin (b) Formation of higher order gel network of mucin on the oral mucosa

1.4.2 Interfacial and Surface Rheology

The surface of salivary fluid contains high levels of surface active molecules, the majority of which are proteins (Proctor et al. 2005). This affects the way saliva adsorbs onto the oral surface as well as how it carries tastants and other volatiles, which are often also surface actives, to the taste bud cells effectively (Janssen et al. 2007, Salles et al. 2011). Surface tension of a fluid is characterised as the ability of the liquid to resist an external force. This is important in saliva as it dictates how stable a salivary film is and affects surface protective capability (Preetha and Banerjee 2005, Rossetti et al. 2008). Dilatational modulus of saliva, or the variation of a liquid's area when in constant shape indicates the softness of the protein layer at the surface of saliva (Vijay et al. 2015). This can provide a measure of the ability of saliva to absorb taste molecules and transport them. Dilatational modulus may also demonstrate how well saliva allows taste molecules to diffuse through the salivary film, on the tongue (Rossetti et al. 2008).

The surface wetting ability of saliva is important as saliva must adhere to surfaces in the oral cavity in order to provide a protective, lubricating film. The contact angle of a droplet of saliva is the measurement of surface wetting capability. Contact angle is the angle of the surface of the liquid, at the point it contacts the solid surface (Vissink et al. 1986). By measuring contact angles of saliva, it is possible to determine the surface wetting properties and thus, its lubricating potential. Stimulated saliva has a higher contact angle than un-stimulated saliva and therefore, has reduced surface wetting capability (Vijay et al. 2015).

1.4.3 Viscosity

There is a certain level of disagreement within the literature regarding the viscosity of saliva. This is largely due to differences in techniques used to measure viscosity and in testing conditions, such as shear rate and temperature. The use of cone and plate rheometry, gives a measure of resistance to shear and is common for measurements of salivary viscosity (Rantonen and Meurman 1998). Other studies have used oscillatory viscometers and showed the viscosity of saliva to be 1.09 mPa.s for un-stimulated and 1.05mPa.s for chewing stimulated, at an oscillation of 500 Hz (Inoue et al. 2008). However another study using a similar oscillatory technique obtained values of 1.5–1.6 mPa s over a shear rate of 1–300 s−1 for un-stimulated saliva (Van der Reijden et al. 1993). Ueno et al. used cone and plate viscometer at a shear rate of 90 (1/s), speed 12 (rpm) and found an un-stimulated salivary viscosity of 3.91mPa.s, for healthy adults

(Ueno et al. 2014). Another study, using the same viscometer, obtained values of 3.8 to 8.8 mPa s at shear rate of 90 s−1 for un-stimulated saliva (Rantonen and Meurman 1998). Increased salivary viscosity has been linked with oral malodour and periodontal disease (Ueno et al. 2014). Since saliva has a highly visco-elastic surface, at the air-surface interface, this can affect viscosity measurements. As such, considering the influence of the surface layer of saliva when measuring viscosity is therefore important (Waterman et al. 1988).

1.5 Effect of Saliva on Taste

The entire oral mucosa is bathed in saliva. Saliva in the mouth aids with transduction of tastant molecules. It also solubilises them, facilitating the movement of taste molecules to the taste pore where they may bind to type ii receptor cells, for bitter, sweet and umami tastes, or access ion channels, for salt and sour tastes (Bradley et al. 2003).

1.5.1 Effect of Xerostomia or Hyposalivation on Taste Function

Studies investigating the effect of xerostomia on taste function have generally shown decreased taste sensitivity in dry mouth patients, indicating an important role for saliva in taste function (Temmel et al. 2005, Negoro et al. 2004, Hershkovich and Nagler 2004). One study investigating taste loss in xerostomic patients, with total or near total loss of salivary gland function, found that patients do not experience reduced taste sensitivity to suprathreshold concentrations of basic tastants (Weiffenbach, Fox and Baum 1986b). However, the study did conclude that detection thresholds were increased in the patient group (Weiffenbach et al. 1986b). Also, the study involved a small group of seven patients, all of whom were suffering different pathologies and degrees of disease. The study also compared taste sensitivity of the 7 patients to large groups of healthy controls, N=144 for sucrose thresholds (mixed age and sex), N=69 for citric acid (female only), N= between 33 and 60 for quinine and sodium chloride (age matched to each patient +/-10years). This method of comparing to controls may have impacted the results, as age and sex effects on taste may not be limited to bitter, salt and sour tastes respectively. Also, age matching to within ten-years does not guarantee to control for age related changes in taste, since changes in taste central processing can occur as early as middle age (Green et al. 2013). Hyposalivation (un-stimulated salivary flow <0.1ml/min) and even self-perceived dry mouth, in the absence of reduced flow rate, have been linked with self-reported poor taste ability (Ikebe et al. 2002).

Removal of the major salivary glands results in pathological changes in the taste tissue and significant decrease in taste sensitivity (Matsuo, Yamauchi and Morimoto 1997, Nanda and Catalanotto 1981). Excision of the submandibular/sublingual salivary glands led to significantly reduced chorda tympani responses to salt, sour, sweet and bitter tastants in rats (Matsuo et al. 1997). Similarly, rats with excised submandibular/sublingual glands have also been shown to exhibit an immediate reduction in behavioural responses to bitter, sour and salt taste compounds (Catalanotto and Sweeney 1973). Submandibular/sublingual sialoadenectomy has also been shown to affect fungiform papillae, with a reduction in numbers and altered morphology but no changes were evident in the circumvallate papillae (Morris-Wiman et al. 2000).

1.5.2 Diffusion of Tastants to the Taste Bud

Taste buds situated in the posterior part of the tongue are constantly bathed in secretions from the von Ebner salivary glands, while taste buds in the anterior tongue are bathed in mixed whole saliva (Gurkan and Bradley 1987, Gurkan and Bradley 1988). Saliva aids diffusion of tastants firstly by solubilising them since taste molecules must be in solution to reach taste buds (Pedersen et al. 2002). Secondly, by facilitating their movement to the taste pore where they may bind to receptor cells (De Almeida et al. 2008, Bradley et al. 2003). A taste solution flowing across the tongue must diffuse through a 10µm layer to reach taste receptor cells (Matsuo 2000). As a fluid enters the oral cavity it displaces part of the salivary pellicle but the layer in direct contact with the tongue is less easily displaced due to surface binding properties of the mucosal pellicle (DeSimone and Heck 1980). The tastant, therefore, can only reach the receptor cell by diffusion. Measurement of neural responses to taste solutions, in rats, have demonstrated that salivary flow rate directly affects taste responses with reduced flow rate equating to a reduced response magnitude (Smith and Bealer 1975). As such, reduced volume of saliva may be a factor transduction of tastants. Water in saliva acts as a diffusing medium for tastants but the taste diffusing capacity of saliva may also be affected by the interaction between salivary components and taste molecules (Matsuo 2000). High interfacial tension of saliva can reduce dissolution, since it may reduce spreading of taste molecules - as shown in drug dissolution studies (Efentakis and Dressman 1998).

Further, a highly viscous saliva might impede tastant interaction with receptors, as it may act as a physical barrier to the taste receptor cells. Indeed, solubility of

hydrophobic compounds has been shown to be reduced by a high viscosity dissolution medium (Braun and Parrott 1972). Therefore, highly viscous saliva may also reduce solubility of taste molecules particularly hydrophobic compounds such as certain bitter tastants (Braun and Parrott 1972). However, saliva is not a simple viscous fluid like polysorbate solutions. Dissolution in a complex fluid, such as saliva, also depends on inter-molecular interactions with the mucous gel and penetration of water within the gel, which allows for solubilisation of the taste molecule (Su et al. 2003). The bulk rheology of saliva may also be less important for diffusion compared to the rheology within pores of the mucin gel - termed the micro rheology (Sigurdsson, Kirch and Lehr 2013). Studies looking at the rheology of pig gastric mucin (PGM) have shown that the gel network is heterogenous, with large pores in which the viscosity is around 100 times lower than the bulk viscosity of PGM gels (Celli et al. 2005). Also, the viscosity of various polysaccharide gums has been shown to have no effect on sweetness perception of sweet taste molecules, therefore viscosity may be less important for taste function than other rheological qualities of saliva (Malkki, Heinio and Autio 1993).

Studies investigating diffusion, through gastric mucin gels, have shed light on possible effects of saliva on tastant diffusion. For one, smaller molecules may become trapped within mucin networks, while larger molecules, such as sugars, can diffuse more easily (Lafitte, Thuresson and Söderman 2007). Diffusion is also dependent on hydrophobic interactions between tastant molecules and hydrophobic portions of the mucin peptide backbone (Larhed et al. 1997). As such, greater lipophilicity and hydrophobicity of a compound leads to increased mucin interaction and a reduced diffusion coefficient (Larhed et al. 1997, Norris and Sinko 1997, Matthes, Nimmerfall and Sucker 1992). Additionally, particle charge also affects diffusion through mucin networks. Highly charged particles diffuse slowly while un-charged particles diffuse much more rapidly, through the mucin network (Lieleg, Vladescu and Ribbeck 2010). Counterions including Na^+, eliciting salt taste, can also bind to mucins via electrostatic interactions (Crowther and Marriott 1984). In drug studies, reduced diffusion can equate to a barrier to absorption, however more recently it has been shown that this mechanism may facilitate retention of compounds in the oral cavity (Boddupalli et al. 2010). For this reason, when investigating salivary mechanisms for taste loss, it is important to investigate mucins as they may provide insight into diffusion capacity of saliva.

Interestingly, age related reduction in bitterness taste thresholds have been shown to be greatest for compounds with less lipophilicity, therefore, a more lipophilic compound is tasted less well by older adults (Schiffman et al. 1994a). Perception of sweet tastants and odour thresholds have also been shown to relate to lipophilicity of the compounds (Deutsch and Hansch 1966, Greenberg 1979). Detection of odorants can also be affected by saliva since retro-nasal olfaction via the mouth requires an interaction between the odour molecule and saliva. This facilitates retention of the odorant in the mouth after swallowing allowing the exhaling breath to transport the odour molecules to the nasal cavity (Salles et al. 2011). In this thesis, it was therefore hypothesised that reduction in salivary mucin in older adults causes reduced interactions with lipophilic tastants/odorants such that the tastants/odorants may be less well retained in the mouth with a subsequent reduction in taste perception.

Increasing muco-adhesive properties of drugs may improve the bioavailability by increasing the retention time of drug carriers and so allowing for local delivery of drug compounds (Lehr 1994). Recently, the effect of muco-adhesion on taste molecules has also become apparent. Since saliva is already highly muco-adhesive, the idea that certain tastants, either highly charged or hydrophobic in nature, could interact with mucins suggests that muco-adhesion could play a role in retention of tastants on the tongue with subsequent effects on perception (Cook et al. 2017). Studies investigating polysaccharide muco-adhesive carriers, for taste molecules, have shown that certain polysaccharides can facilitate persistence of the perception of sweet taste molecules. This may be due to enhanced retention of taste molecules, in proximity to the taste buds (Malkki et al. 1993).

Table 1-2 summarises results from multiple studies investigating the effects of polysaccharides on taste and flavour. It is evident that the effect is variable and dependent on which polysaccharide is used. Muco-adhesion may, in part, explain the effects, since these studies have used known muco-adhesives including pectin, sodium alginate and carboxylmethyl cellulose (Cook et al. 2017). Carboxymethyl cellulose (CMC) based saliva substitutes have been shown to be ineffective in improving taste function in xerostomic patients (Temmel et al. 2005). This may indicate that CMC does not facilitate tastant diffusion well which correlates with results from many of the *in vitro* studies summarised in Table 1-2 (Temmel et al. 2005). CMC is an ionic polysaccharide and has sodium ions attached to its negatively charged carboxylic

groups (Cook et al. 2018). An anion effect may prevent subsequent enhancement of taste intensity, with muco-adhesion of sodium-CMC mixtures since CMC already contains sodium and acts as the anion to polarise taste cells (Cook et al. 2018). Hydroxypropyl methylcellulose (HPMC) has also been shown to cause reduced taste perception, however it is non-ionic and thus does not have the same interaction with tastants that saliva does (Cook, Linforth and Taylor 2003). Muco-adhesion may therefore have modulatory effect on taste and flavour perception.

Table 1-1 Adapted from (Cook et al. 2017) A summary of the effect of various polysaccharides on taste and flavour perception

Study	Polysaccharide(s) used	Effect on Taste or Flavour
Mouthfeel and flavour of fermented whey with added hydrocolloids (Gallardo-Escamilla, Kelly and Delahunty 2007)	Propylene glycol (PG) alginate Carboxylmethyl cellulose (CMC) High-methoxy pectin Xanthan Gum (XG)	CMC and PG alginate ↑ sweetness and ↓ acidity compared to other matrixes
Volatile flavor analysis and sensory evaluation of custard desserts varying in type and concentration of carboxymethyl cellulose (van Ruth, de Witte and Uriarte 2004)	CMC with varying viscosity grades and concentrations used	↓ sweetness perception and ↑ the in-nose total release and maximum intensity of ethyl butyrate, ethyl 3-methylbutanoate, ethyl hexanoate
Influence of the texture of gelatin gels and pectin gels on strawberry flavour release and perception (Boland, Delahunty and van Ruth 2006)	Pectin Gelatin	Increased gel rigidity ↓ in-nose release rates, perception of odour, strawberry flavour and sweetness but ↑ total release and intensity for hexanal, ethyl butanoate, ethyl 3-methylbutanoate and ethyl hexanoate. Pectin gels ↑ time intensity and maximum intensity compared to gelatin gels for all aromas.
Viscosity and flavour perception: Why is starch different from hydrocolloids? (Ferry et al. 2006)	Hydroxypropylmethyl cellulose (HPMC) Starches: wheat, waxy maize, and modified waxy maize	HMPC ↓ salt and basil flavour perception compared to all starches. Waxy maize starch ↓ salt and basil flavour compared to other starches.
Relating the microstructure of pectin and carrageenan in dairy desserts to rheological and sensory characteristics (Arltoft, Madsen and Ipsen 2008)	Pectin - with differing Ca^{2+}reactivities	Sweetness and vanilla perception were unaltered.
Flavor release and sensory characteristics of o/w emulsions. Influence of composition, microstructure and rheological behaviour (Arancibia et al. 2011) Impact of structural differences on perceived sweetness in semisolid dairy matrices (Arancibia, Costell and Bayarri 2013) Colour, rheology, flavour release and sensory perception of dairy desserts. Influence of thickener and fat content (Arancibia et al. 2015)	CMC Modified starch	CMC ↓ overall flavour and sweetness perception compared to starch samples.
Influence of carboxymethyl cellulose and sodium alginate on sweetness intensity of Aspartame (Han et al. 2014)	CMC Sodium alginate (SA)	CMC ↓ sweetness perception of aspartame. SA did not have an effect on sweetness perception.
Mucoadhesive polysaccharides modulate sodium retention,	CMC	↓ salt intensity but ↑salt retention on the oral mucosa

release and taste perception (Cook et al. 2018)		

1.5.3 Salivary Growth Factors and Proteins Required for Taste

Saliva has antibacterial and lubricating properties and provides growth factors for renewal of taste buds (Morris-Wiman et al. 2000, Mese and Matsuo 2007).

Carbonic anhydrase (CA) VI in saliva is also associated with taste function (Shatzman and Henkin 1981). CAs are zinc metalloenzymes which catalyse the hydration of carbon dioxide into bicarbonate and protons (Chandrashekar et al. 2009). As a result, CA in saliva is associated with effective buffering and sensory detection of carbonation (Chandrashekar et al. 2009). A study investigating the *rs2274327* single nucleotide polymorphism of the CAVI gene, found that individuals with allele T and genotype TT were more likely to have lower salivary buffering capacity (Peres et al. 2010). In addition, individuals with the GG variant of polymorphism *rs2274333* (A/G) of CAVI exhibit reduced taste sensitivity to TAS2R38 bitter agonists, PROP/PTC (Padiglia et al. 2010, Calò et al. 2011). Although in a larger, ethnically heterogenous cohort, the same association could not be made (Feeney and Hayes 2014). Nonetheless, saliva from individuals with the AA variant increases taste cell proliferation and was positively associated with maintenance and development of fungiform papilla, demonstrating a functional effect of the A/G SNP on CAVI (Melis et al. 2013b). CAVI is released by acinar cells of the submandibular and parotid glands, as well as the von Ebner minor salivary glands, the secretion of which bathes the taste buds in the circumvallate and foliate papillae (Leinonen et al. 2001, Parkkila et al. 1990, Thatcher et al. 1998). It may have a role in maintaining and development of healthy taste buds since it holds functional similarities to nerve growth factors (Henkin 1978).

Zinc deficient or low zinc diet rats have reduced salivary CA activity (Komai et al. 2000). This may be due to the fact that zinc is required for normal CA synthesis, acting to stabilise the protein conformation as in other metalloenzymes (McCall, Huang and Fierke 2000, Shatzman and Henkin 1981). Taste dysfunction is often reported as a side effect of carbonic anhydrase inhibitor medications (Miller and Miller 1990). Further, patients with taste dysfunction have been shown to have approximately 20% of the amount of salivary CAVI than normal taste function subjects (Shatzman and Henkin 1981). However, this was in a small study group (16 healthy and 4 taste dysfunction

patients) and there were no discriminations made for the cause of taste loss which may have varied amongst patients. Subsequent studies have found no difference in salivary CAVI between taste dysfunction and normal taste subjects, and even an increased level in saliva from individuals with low sweet taste sensitivity (Schoeps et al. 2016, Feeney and Hayes 2014, Rodrigues et al. 2017). Zinc treatment has proven effective in improving taste disorders in zinc deficient patients indicating a role for zinc and/or CAs in maintaining normal taste function (Tanaka 2002). Additionally, zinc supplementation in patients with abnormal taste bud morphology and low salivary CAVI led to improved taste function and normalisation of CAVI levels. Taste bud morphology also returned to normal following zinc supplementation. However, this was also shown in a small study group and four out of fourteen patients did not respond to zinc treatment at all (Henkin, Martin and Agarwal 1999).

Recent work by Henkin et al. has shown that sonic hedgehog (Shh) may also play a role in taste function by acting as a growth factor for taste buds (Henkin and Abdelmeguid 2017). Shh has been found in parotid saliva, with reduced levels in the saliva of taste disfunction patients (Henkin et al. 2017). Shh has previously been shown to be important in basal cell renewal activity in taste buds enhancing cellular proliferation (Miura et al. 2004). Furthermore, transgenic ablation of Shh in sensory neuronal cells of mice led to almost complete loss of taste receptor cells (Lu et al. 2018). Pharmacological blocking of Shh also led to loss of taste bud cells with pharmacological enhancement of Shh leading to regeneration (Lu et al. 2018). A study by Azofeifa et al. (2017) showed that inhibition of Shh led to loss of differentiation of new taste bud cells but did not affect taste cell proliferation (Castillo-Azofeifa et al. 2017). Additionally, this was dependent on removal of Shh from both sensory neurons and epithelial taste precursor cells since loss of either one source had minimal effects on numbers of taste bud cells (Castillo-Azofeifa et al. 2017). Loss or alteration of taste function is also seen in patients taking medications that inhibit Shh (Rodon et al. 2014).

There is some disagreement around the effect of CAVI on taste and studies on Shh are relatively recent and require further investigation. However, the work of Henkin et al. and others demonstrate the potential effects of salivary growth factors on maintenance of the taste system. Further research into salivary growth factors is required to gain insight into their potential effects on taste loss. This would be particularly interesting in terms of age-related taste loss since most previous studies have focussed on taste loss in

general from mixed pathologies. Therefore, the effect of salivary CAVI on age related taste loss is largely unknown.

Odorant binding proteins have been shown to occur in the nasal cavity and can bind to hydrophobic volatile odour molecules. Such proteins may assist with transportation of odour molecules through the mucous layer in the nasal cavity to reach odour receptors. These proteins can also aid smell function by creating a complex with odour molecules with improved recognition in olfactory receptors (Pelosi 1994). Rat models have been used to further demonstrate the mechanism of odorant binding protein action. It has been shown that there are surface active as well as bulk OBPs in the nasal mucosal fluid. The surface-active proteins capture odorant molecules and transport them to the olfactory bulb where they are rapidly offloaded onto receptors. This quick action offloading is key to the action of OBPs as it allows quick detection of smells and multiple smells to be rapidly detected. This is highly important since humans are frequently subjected to a multitude of diverse aromas (Borysik et al. 2010). Recently, evidence has pointed towards tastant binding proteins, which may act in a similar way to OBPs, to facilitate rapid detection of different flavours and tastes. One possible candidate for a TBP is Ebnerin, a protein released by Von Ebner glands in the mouth (Figure 1-7) (Li and Snyder 1995). This protein is similar in structure to OBPs in that it is hydrophobic binding and so could potentially bind volatile flavour molecules to assist their transport through the salivary pellicle to the taste pores (Schmale, Holtgreve-Grez and Christiansen 1990). Using animal models, studies have also shown that Ebnerin may assist taste function by binding to growth factor hormones delivering them straight to the taste bud to aid in regeneration of healthy taste cells (Li and Snyder 1995). Decreased levels of Ebnerin, or other taste-binding proteins as a function of ageing could therefore provide another mechanism for age-related taste loss.

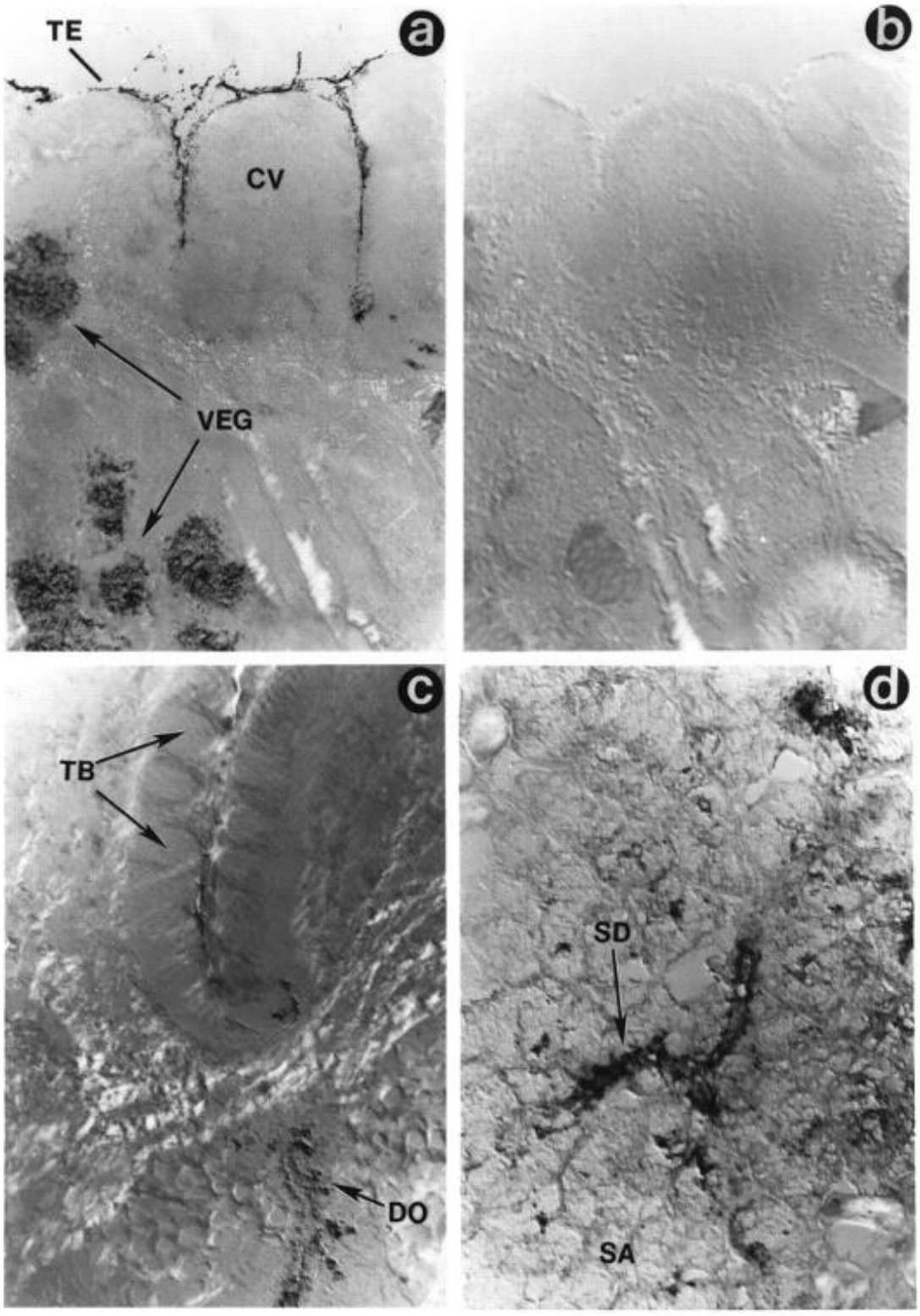


Figure 1-7 (Li and Snyder 1995) Immunohistochemical localization of Ebnerin in rat tongue. von Ebner's glands (VEG), Taste buds (TB), Circumvallate papillae (CV) Surface of tongue epithelium (TE), Epithelial cells of secretory ducts (SD), secretory acini (SA).

1.5.4 Salivary pH

Additionally, salivary pH has a significant impact upon sweet taste sensation. Rat tongue, adapted to saliva, had double the magnitude of chorda tympani nerve response to sweet taste stimulation, than that of xerostomic rats (Matsuo and Yamamoto 1990). This could be correlated with increased pH and could also be replicated using artificial saliva, containing bicarbonate at pH8-10 (Matsuo and Yamamoto 1992). In human studies, a significant correlation between increased resting salivary pH and heightened sweet taste sensitivity has been also shown (Aoyama et al. 2017).

1.6 Chemosensory Decline in the Elderly Population

1.6.1 Effects of Ageing on Taste, Olfaction and TRP Sensitivity

Advancing age is linked with the decline of taste acuity, often coupled with decreased olfactory sensation, particularly retro-nasal olfaction which occurs via the mouth (Stevens and Cain 1986). Both senses appear to decline noticeably at similar rates over the human lifespan usually from the age of 60 (Ng et al. 2004, Schiffman 1993) but possibly even as early as middle age (45 to 54 years old) (Green et al. 2013). The loss of either sense usually occurs concurrently with the other, suggesting that an individual experiencing loss of taste could in fact have diminished olfactory capacity or vice versa (Kaneda et al. 2000). It is thought that olfactory decline is greater than taste losses with age (Stevens, Bartoshuk and Cain 1984, Murphy et al. 2002).

The senses of smell and taste are both governed by receptor cells in the olfactory epithelium or taste bud respectively. Ultimately, signals from these cells sent upon stimulation from odorant/tastant molecules are sent to the thalamus in the brain where they intertwine to give the individual sense of flavour (Frank, Vanderklaauw and Schifferstein 1993). A loss of taste sensitivity can have serious implications on the health and quality of life for an elderly individual (Wylie and Nebauer 2011). Their nutritional status may be impaired, as a result of reduced food intake, because of an inability to taste and therefore experience hedonic aspects of eating (Schiffman 1997). Malnutrition is a significant health risk for older adults with previous studies finding that between 5 and 10% of independently living older individuals have nutritional deficits (Brownie 2006). These numbers rise to between 30 and 60% percent of hospitalised or institutionalised patients and up to 85% amongst older adults in residential homes (Brownie 2006). Weight loss, due to ageing, is associated with greater risk for mortality compared to individuals who maintain their weight into older age (Newman et al. 2001, Potter, Schafer and Bohi 1988). Older adults may also increase salt intake, in an attempt to heighten flavour in their meals (Stevens et al. 1991), or resort to eating more sweet foods (Gilmore and Murphy 1989). This can have clear effects upon health and disease status especially in adults over 60 years for whom the risk of diet related disorders such as heart disease, high blood pressure, stroke and diabetes reaches a peak (Amine et al. 2003, Prince et al. 2015). Salt intake is directly linked with increased blood pressure and cardiovascular disease while high intakes of added sugar is associated with fatty liver disease, dyslipidaemia, insulin resistance,

hyperuricemia and cardiovascular disease (Stanhope 2016, He and MacGregor 2009). Loss of sense of smell can also have health implications for an elderly individual as they may not be able to detect differences between fresh and out of date foods or may fail to notice a gas leak (Boyce and Shone 2006).

TRP receptor channels are found all over the oral mucosa including the tongue surface and are responsible for sensations such as heat, cooling, tingling and burning that are evoked in the mouth when eating or drinking (Ramsey, Delling and Clapham 2006). All these sensations may be involved in taste and enjoyment of food. A reduction in sensitivity of TRP channels in older adults could therefore influence the hedonic aspects of eating (Fukunaga, Uematsu and Sugimoto 2005). Studies of perception and thresholds of menthol, a TRPM8 antagonist, have identified increased taste thresholds and reduced responses to repeated exposure in older adults compared to younger subject groups (Murphy 1983). This suggests that oral sensations as well as taste acuity are affected by advanced age and adaptation mechanisms to oral stimuli may also be less functional. In contrast, capsaicin, a TRPV1 antagonist producing heat and tingling sensations in the mouth, does not appear to have a reduced effect on aged subjects even in those who have loss of basic taste sensation (Fukunaga et al. 2005). This may indicate the potential usefulness of TRP receptor stimulants to evoke oral sensations which could contribute to making food more pleasant for older individuals who have lost their taste ability. Indeed, several previous studies have investigated the use of trigeminal stimulants, such as capsaicin and menthol, as compensatory methods to improve hedonics of food products in older adults with sensory loss. The results have been conflicting thus far perhaps due to heterogeneity in sensory capabilities and food preferences amongst older adults (Forde and Delahunty 2002). However, for some older adults, addition of threshold concentration of capsaicin to tomato products may increase liking of a food product by increasing the level of or altering the sensation elicited during eating (Kremer et al. 2007a, Forde and Delahunty 2004). On the other hand, addition of capsaicin to custard or menthol to yoghurt may have a negative effect on liking in older consumers (Koskinen, Kälviäinen and Tuorila 2003, Kremer et al. 2007b). The compound and food specific effects of TRP agonists on liking may be due in part to the novelty of food products used in testing. For example, while some older adults are likely to be familiar with the sensation of chilli or heat in a savoury food product such as tomato juice, the addition of menthol to yoghurt or capsaicin to custard may elicit a degree of neo-phobia in consumers (Koskinen et al. 2003). This may

particularly apply to older adults as studies investigating neo-phobia in ageing have found a reduced willingness to try new foods in adults aged >65 compared with younger adults (Bäckström, Pirttilä-Backman and Tuorila 2003, Tuorila et al. 2001).

1.6.2 Perception changes in taste with age

Many studies have shown that ageing results in increases in thresholds of basic tastes especially bitter and sour tastes. Older adults are less able to discriminate between tastes as well as finding tastes less intense than their younger counterparts (Yoshinaka et al. 2016, Nilsson 1979, Mojet, Heidema and Christ-Hazelhof 2003, Mojet, Christ-Hazelhof and Heidema 2001, Nordin et al. 2007). There have also been differences shown for salt taste with higher thresholds in older adults compared to younger adults, adolescents and children (Jiang, Jung and Lee 2016). In a review by Methven et al (2012), 69 studies were analysed to combine the results from taste testing studies in older adults. Overall, it was shown that older adults displayed increased detection thresholds in almost all the studies (94%). Over 60% of studies found decreased suprathreshold sensitivity in older adults. More than 80% of the studies found increased thresholds for salt and sour tastants while 70% showed increased bitter and sweet thresholds in older adults. There were fewer studies which had investigated umami taste thresholds but in those that did increased thresholds were generally shown in older adults. It was apparent from the review that there are significant discrepancies amongst the literature for taste and ageing, largely due to differences in testing method, sample size, compounds used and gender distribution of participants (Methven et al. 2012).

The findings from some of the studies which have investigated taste loss in ageing are presented below. As described above, umami taste appears to be reduced with age, with increased thresholds for various glutamate salts (Mojet et al. 2001, Schiffman et al. 1991, Schiffman et al. 1994b). Increased detection thresholds for MSG in foods have also been shown but only in individuals from western countries (Mojet et al. 2001, Schiffman et al. 1991, Schiffman et al. 1994b). Increased threshold and preferred concentrations for l-glutamate have also been shown in an Asian population although only in females (Hayakawa et al. 2008). Addition of IMP to other glutamate salts can restore, partially, taste perceptions for umami in older adults (Schiffman et al. 1991). This may be due to the enhanced binding of L-glutamate to glutamate receptors, in the presence of inosine 5'-monophosphate (Schiffman et al. 1991). Since addition of umami flavour has lesser negative health effects than salt or sugar, this tastant has been the

subject of various intervention studies aimed at improving palatability of food and increasing appetite for older adults (Schiffman et al. 1991, Masic and Yeomans 2014, Schiffman 1998). Furthermore, umami tastants may increase salivary flow and improve oral health (Sasano et al. 2010, Schiffman 1998). Therefore, they could be useful in improving taste function in older adults as taste function is correlated to both hypo-salivation and poor oral health (Sasano et al. 2010, Schiffman 1998).

Bitter taste may be the one of the most susceptible taste modalities to age related depletion since consumption of bitter tasting foods such as vegetables is encouraged for nutritional benefit throughout the lifespan. This may lead to adaptation of bitter taste which does not occur with sweet taste as sweet foods are consumed from birth while bitter tasting foods such as coffee and alcohol are habitually ingested throughout adulthood (Yoshinaka et al. 2016). The same may be true for salt taste. Indeed, in a cohort of Korean individuals, those with the highest salt consumption also had higher salt detection thresholds (Jiang et al. 2016). However, previously this could not be shown in a smaller western population of older adults (Drewnowski et al. 1996). An effect of cognitive decline and the ability to recall salt intake for food diaries in older adults must not be ignored.

There is still disagreement as to which taste modalities are depleted first and to what extent. For example, some researchers have suggested that the sweet taste modality is largely retained with age with less difference in thresholds for sweet taste than for other basic tastes (Mojet et al. 2001). A study on a large group of Japanese subjects found no differences in taste threshold for sucrose between young old (69-71 years, n=996) and young adults (24-32 years, n=70) but a significantly increased threshold from young old to old-old adult (79-81 years, n=949) using whole mouth gustatory testing. Another study showed no differences in just noticeable above threshold taste stimuli, known as Weber Ratios, for sucrose between older (67-77 years, n=24) and younger (18-25 years, n=24) adults (Gilmore and Murphy 1989). Meanwhile others have shown that sweet taste is also reduced with age with a significantly increased threshold for sucrose in older individuals (65-87, n=15) compared to younger (19-26, n=15) (Stevens et al. 1995). It is possible that sweet taste is retained for longer and depletion in sweet taste sensation does not occur until later in life. The Gilmore study had upper age limits of 77, while the Stevens study looked at individuals aged up to 87. In the Yoshinaka study, depleted sweet taste was only evident in those aged 79 and above. Gender differences

may also play a role since the Gilmore study exclusively tested female subjects. Males are generally more susceptible to age related taste loss (Yoshinaka et al. 2016, Mojet et al. 2001, Mojet, Christ-Hazelhof and Heidema 2005), however, some studies have found that in sweet taste ability, specifically, sex differences do not a play a role (Mojet et al. 2001, Yoshinaka et al. 2016).

Similarly, a study investigating taste thresholds of artificial sweeteners in ageing found that older adults had higher forced-choice threshold for aspartame and other sweeteners (n=12 of each age group, 19-24 years and 75-81 years) (Schiffman et al. 1981). Conversely, another study, also using aspartame to measure sweetness perception, found no difference in thresholds with age, determined by pair comparison between younger participants (19-33, n=22) and older (60-75, n=21) (Mojet et al. 2001). The difference in upper age limits, 81 years compared to 75 years, again highlights the possibility of taste ability for artificial sweeteners being retained until later in life. Furthermore, as discussed below, differences in taste compounds and type of threshold measure chosen can also affect the results of taste or smell test studies.

Taste identification also decreases with age and greater taste confusion is observed in older adults (Doty, Chen and Overend 2017, Nordin et al. 2007). Sour-bitter and bitter-sour confusions are most common and occur more frequently in older adults than younger (Doty et al. 2017). Salt-bitter or bitter-salt confusions have also been shown to occur more frequently in older people compared to younger (Nordin et al. 2007, Doty et al. 2017).

1.7 Methods for Measuring Taste and Olfaction

1.7.1 Traditional Methods for Sensory Analysis

Researchers have previously used staircase methods to analyse taste thresholds and taste discrimination ability of the ageing population (Stevens et al. 1991). This involves presenting a participant with tastant dissolved in water at different concentrations and asking them to note when they perceive a change in concentration. After two correctly identified changes in concentration a lower strength taste solution is given, until the person perceives the reduction in taste. Then an average of the two correctly identified concentrations is taken, to give a threshold value. Three-way forced choice methods are also popular in this kind of research, whereby three samples are presented, one with a certain taste concentration and two without. The participant is asked to identify the sample containing a taste (Ng et al. 2004). Ascending concentrations may be given to assess a threshold for each taste and used alongside a visual analogue scale or multiple-choice questionnaires to rate perceptions of the intensity of the tastant presented.

Taste testing can give the researcher a subjective measure of taste ability and assesses perception of the tastants rather than physiological responses such as taste receptor activation. Furthermore, there is no standardised-universal taste test. This could be one of the reasons for differences in results between studies (Hoffman, Cruickshanks and Davis 2009). Additionally, the older population are largely more heterogeneous in sensory responses than their younger counterparts with greater inter-individual variations in perceptions and responses to taste, smell and oral sensations (Stevens et al. 1995, Forde and Delahunty 2004).

1.7.2 Filter Paper/Edible Film Strips

More recently the use of filter paper disks and edible taste strips has been used to assess taste function (Smutzer et al. 2013, Smutzer et al. 2014, Landis et al. 2009, Smutzer et al. 2008, Berling et al. 2011). This has several benefits including the potential for standardisation between studies and the ease with which strips are transported and stored (Smutzer et al. 2008). In addition, a recently patented taste test (T@sty test™) has been used to assess taste thresholds using taste test edible wafer sheets with pre-cut discs of ascending concentrations of stimulant and blank disks to mimic an alternative forced choice taste test (Feng et al. 2018). However, it remains to be seen what the effect of using such methods are on subjects with reduced salivary flow or dry mouth

since there is no solution in which to transport the tastant to the taste bud. If the subject also has reduced salivary flow or altered salivary rheology, for example very viscous saliva, there may be reduced dissolution of the taste molecules. In the case of dissolving taste strips which require saliva as a dissolution medium dissolution may be incomplete (Desai et al. 2011). This could result in reduced perception, even in cases where there is no actual taste loss. However, some studies have failed to find a difference in taste function between older and younger adults when using the taste strips method (Ogawa et al. 2017). Alternatively, if there is no dilution of the taste molecules due to lack of solubilising medium there may be heightened sensation compared to an individual with normal salivary flow. Therefore, the results may not be reflective of taste ability. Studies by Smutzer et al., investigating use of dissolving strips for taste testing, have shown that the concentrations needed to elicit responses are much lower when using strips compared to taste solutions due to localised presentation of the taste compounds within the oral cavity (Smutzer et al. 2013).

In a study of taste function in Sjögren's syndrome patients taste ability was significantly reduced when the filter paper disk method was used, compared to electrogustometry which uses an electrical current to assess taste responses (Negoro et al. 2004). This may be because of the effects of reduced salivary flow on taste function and suggests that reduced taste in individuals with reduced salivary flow may be due to impaired diffusion of tastants to the taste bud. This is because electrogustometry assesses nerve responses, which were not affected by Sjogren's syndrome (Negoro et al. 2004). Furthermore, several studies have shown that distribution of tastant on impregnated filter papers is not always even, and as such, may result in false negative results (Lawless 1980, Zhao, Kirkmeyer and Tepper 2003b). There may also be an effect of localised testing, since taste function differs depending on the region of the tongue being tested, especially in older individuals (Doty et al. 2016). Taste strips do not allow assessment of whole mouth taste function (Landis et al. 2009, Bartoshuk 1989).

1.7.3 Thresholds Compared to Supra-Threshold and Preference

Taste thresholds are often much lower than the concentrations of tastants found in foods and so measuring taste thresholds may not give an accurate picture of the real life effects of taste loss in older adults (Schiffman et al. 1994b, Kremer et al. 2007b). Foods and meals are made up of multiple tastes and smells therefore measuring perceptions of individual tastes or smells may not reflect ability to taste or smell actual foods (Graaf,

Polet and van Staveren 1994). Detection thresholds in both younger and older adults for umami flavour are higher when MSG or IMP is added to foods than when it is presented in water. This may be due to interaction between umami compounds and other compounds, present in different foods, which may affect the levels of free MSG or IMP (Schiffman et al. 1994b). Reduced ability to detect MSG in some foods such as tomato soup but not in others such as steak has also been shown in older adults. Again, this may be due to different levels of umami compounds already present in different foods (Schiffman et al. 1994b). NaCl thresholds are around seven times greater when NaCl is presented in tomato soup rather than in water regardless of age. However, the ability to detect salt in soup is significantly reduced in older adults (Stevens and Cain 1993). This also highlights the complexity of taste detection in different food products, with multi-sensorial factors playing a role- including olfaction, texture and chemo-sensation.

Some studies have shown that older people have reduced ability to identify flavours and tastes in food products, with lower perceived intensities of high concentrations of bouillon, tomato juice and orange juice flavours as well as sugar and strawberry flavour in yoghurt. However, at low concentrations, perceptions are similar to those of younger adults (Graaf et al. 1994).

Additionally, there are differences in threshold and suprathreshold sensitivity between old and young adults. Threshold is usually defined as the lowest concentration required to elicit a response (Keast and Roper 2007). Suprathreshold refers to concentrations above threshold level and is usually used to test perceived intensities of tastant concentrations above the level of detection (Mojet et al. 2005, Keast and Roper 2007). A positive correlation between threshold and supra threshold intensity perceptions has been shown for older adults but not in younger adults (Mojet et al. 2005, Mojet et al. 2003). Therefore, younger adults do not necessarily perceive intensity of concentrations as higher or lower just because their detection threshold is higher or lower. However, in older adults, a reduced detection threshold is linked with reduced intensity ratings of tastants at supra threshold level (Mojet et al. 2005, Webb et al. 2015). This may be because of the presence of multiple taste transduction pathways which are dependent on concentration of the taste compound (Keast and Roper 2007). For example, for quinine and saccharine detection more than one pathway is involved. These amphiphilic tastants activate GPCR taste receptors as well as diffusing through the cell membrane to directly

activate G proteins and trigger secondary messenger signalling (Peri et al. 2000, Naim et al. 1994).

As such, some studies have not found age related differences in taste when comparing supra threshold concentrations of certain tastants (Weiffenbach, Cowart and Baum 1986a, Cowart 1989). This may be because of the inverse relationship between threshold detection and supra threshold intensity for certain tastants, shown for example with quinine (Keast and Roper 2007) and for salt and umami taste (Mojet et al. 2005). Quinine is known to inhibit TRPM5/TRPM4 calcium response currents at concentrations above 100μM as seen in whole-cell patch-clamp experiments using TRPM5/M4 transfected HEK293T cells (Talavera et al. 2008). TRPM5 and TRPM4 are vital components of the taste transduction pathway for bitter compounds, such as quinine, providing a mechanism for reduced taste intensity at suprathreshold concentrations (Banik et al. 2018). As such, the fact that younger adult's sensitivity to suprathreshold concentrations is reduced, even when the threshold detection level is greater, may not be evident in older adults who experience taste loss. Thus, it may seem that older adults with taste loss are perceiving suprathreshold concentrations of tastants at the same level as younger adults when in fact there are age related alterations in the pathways being activated for detection of higher tastant concentrations.

1.7.4 Compound Specific Taste Loss in Ageing

There are apparent differences in elderly taste ability when different compounds which elicit the same taste are used for testing i.e. a compound specific reduction in taste loss (Cowart, 2011) (Mojet et al. 2003). A study illustrating this found that although urea and caffeine both elicit a bitter taste response, there was little taste loss in the older subject group compared to the younger for urea. For quinine, however, a significantly higher average threshold was apparent (Cowart, Yokomukai and Beauchamp 1994). This may be related to the fact that there are over 30 different bitter taste receptors on cells found on taste buds, each thought to be specific for one or more bitter compounds (Matsunami et al. 2000). So, it is possible since quinine and urea are detected via different receptors that changes in quinine receptors over time results in depletion of the sensitivity to this compound. Similarly, differences have been shown for taste thresholds of different sweeteners, despite them stimulating the same receptors on the taste buds (Schiffman et al. 1981, Mojet et al. 2001). This may be because of multiple

taste pathways involved in sensing non-nutritive sweeteners since they are also capable of stimulating bitter taste sensations.

1.7.5 Electrogustometry

Electrogustometry (EGM), is a popular measure of taste function in Japan, employing application of an electrical current to a localised area of the tongue (Berling et al. 2011). Free hydrogen ions at the anode cause acidification of saliva within the localised area being tested and this activates a sour taste response (Herness 1985). The method may not be reproducible between studies since there may be a variable number of hydrogen ions liberated at the anode creating a variable taste response. In addition, the size of electrode used impacts upon thresholds (Stillman et al. 2003, Ajduković 1984). Also, the method does not allow for taste discrimination testing and can only test responses to one stimulus, a sour taste.

It is not clear whether the elicited response is a taste specific response or whether trigeminal stimulation also occurs due to direct stimulation of nerve endings surrounding the taste buds (Stillman et al. 2003). Some studies have shown little correlation between sour taste stimulation and EMG response indicating that EMG may not be solely a sour taste response (Murphy, Quiñonez and Nordin 1995, Ellegård et al. 2007). However, studies which measure EMG responses compared to perceptions of individual basic tastants have shown that EMG responses do correlate with taste perception in general with no specificity for any one taste (Berling et al. 2011, Ellegård et al. 2007). Where EMG has been used in patients with CV nerve damage, the thresholds shown for tactile nerve responses were in a different range to those which occur from taste stimuli. As such, responses to EMG stimulation, in the appropriate range for taste stimuli, are likely to be taste specific (Grant et al. 1987). EMG can measure wide ranges of detection thresholds and thus may be able to detect slight changes in function of the taste system (Stillman, Morton and Goldsmith 2000). Since the test requires the patient to identify when a stimulation has occurred, and usually involves spatial testing in which the current is applied to one side of the tongue and not to the other, cognitive ability is also a factor. As such, the level of a participants understanding of the test can also affect results (Stillman et al. 2000).

1.7.6 Effect of Tongue Region Tested

There are also age-related differences in taste acuity depending on the tongue region being tested (Doty et al. 2016, Nordin et al. 2007). Age related reduction in taste function is generally reported to be most significant in the anterior tongue, regardless of the compound being tested (Doty et al. 2016, Nordin et al. 2007). This may be due to the fact that younger people have greater sensitivity to taste at the front of the tongue, compared to other tongue regions (Doty et al. 2016). For example, when using EMG as a measure of taste function, there is greater sensitivity to stimuli at the tip of the tongue, in young healthy adults (Salata, Raj and Doty 1991). Conversely, a recent study using EMG with older and younger patients found that responses in the anterior tongue were not significantly different between age groups but younger adults had significantly greater responses than older adults when tested on the posterior tongue (Ogawa et al. 2017). This may be because, relative to other tongue regions, the front of the tongue retains more taste sensitivity. However, when comparing older to younger adults - who display heightened sensitivity in this region, the relative taste response decline appears most pronounced in the anterior tongue (Doty et al. 2016). In the present study, whole mouth measures of taste were assessed thus reducing the potential impact of localised testing.

1.8 Objective Measures of Taste Changes in Age

1.8.1 Functional MRI

Functional MRI (fMRI) imaging studies have recently been used for assessing objective taste function (Haase, Cerf-Ducastel and Murphy 2009). Greater activation of the thalamus, hippocampus, caudate tail, para-hippocampal gyrus, postcentral gyrus, precentral gyrus, and cerebellum was observed in younger adults in response to basic taste stimuli (Jacobson, Green and Murphy 2010). In older adults, more robust activation in the emotion, motivation and reward areas of the brain following taste stimulation was evident compared to younger adults (Jacobson et al. 2010). Additionally, more areas of the brain were activated in older adults upon taste stimulation. The age effect was greatest for bitter taste and lesser for sweet, in line with gustatory testing studies which have shown greatest age-related taste losses for bitter taste and least for sweet (Gilmore and Murphy 1989). Activation of the insula during taste stimulation was consistent between the two age groups (Jacobson et al. 2010). These results indicate that greater brain activation may be required for older adults to sense tastants, compared to younger. This has been labelled the "compensation effect" in which reduced capacity of brain function leads to greater activation required to process information (Cabeza et al. 2002).

Further studies have shown greater activation of the bilateral anterior cingulate, lentiform nucleus, putamen, caudate and right precentral gyrus in younger adults (19-26 years old) compared to middle aged (45-54 years old) during sweet and bitter taste stimulation (Green et al. 2013). Also, there was greater activation in the insula and lentiform nucleus regions during sweet taste stimulation in younger adults compared to middle aged indicating that changes to the taste system may begin in middle age (Green et al. 2013).

Additionally, older adults show reduced brain activation in the amygdala in response to increasing taste concentrations compared to younger adults regardless of taste type (Hoogeveen et al. 2015). Furthermore, reduced activation of the medio-dorsal nucleus of the thalamus has been shown in older adults compared to younger during taste stimulation. This may indicate reduced attention to stimuli in older adults (Hoogeveen et al. 2015). While fMRI provides insight into the neural responses to tastants in older adults and is objective, it does not give information about changes in the peripheral taste

system which affect subsequent transmission of taste signals to the brain. If an older adult does not respond to a taste compound at the taste receptor level in the same way as a younger adult would it stands to reason that activation in the brain would be altered. Thus, it is still important to investigate in mouth mechanisms for taste loss to provide insight into what changes occur in the taste system before the neural taste message is sent to the brain.

1.8.2 Neural Studies in Animal Models

Electrophysiological models involving use of electrodes to measure nerve responses to taste stimuli in animals can be used as an objective measure of taste (Sako and Yamamoto 1999, Ninomiya et al. 2000, Zhang et al. 2003, Lundy Jr and Contreras 1997). The technique has been used in few cases in humans because of the invasive nature of the procedure. However, during otological operations, the chorda tympani is exposed and thus accessible to record nerve responses to taste stimuli on an anaesthetised patient. Such studies have shown that chorda tympani responses to sweet, salt, sour and bitter tastants were similar in terms of threshold compared to perception studies on taste thresholds (Diamant et al. 1965, Zotterman 1971). Multiple factors can affect nerve recordings in response to taste including method of stimulus delivery, presence of saliva, temperature of the tongue and use of anaesthetic (Ogawa, Sato and Yamashita 1968, Matsuo and Yamamoto 1990).

1.9 *In vitro* Models of Taste

1.9.1 Heterologous Expression Systems

Functional expression of taste receptors, in cell line models, are another method used to assess the molecular basis for taste. Most frequently, a heterologous expression system is developed using transfection of human embryonic kidney (HEK293) cells with a taste receptor and a G-protein (Ueda et al. 2003). Intracellular calcium responses are then recorded, following staining of cells with a calcium fluorophore, after addition of a taste compound (Tsien, Rink and Poenie 1985). Different methods have been used to measure changes in fluorescence, equating to changes in intracellular calcium levels, including fluorescent microscopy (Chandrashekar et al. 2000), fluorescence imaging plate readers (FLIPR) (Brockhoff et al. 2007, Bufe et al. 2002) and the FLEX station micro-plate reading system (Narukawa et al. 2011, Upadhyaya et al. 2015). Initial studies used cells which had been stably transfected to express the alpha subunit of mouse G-protein 15 (Chandrashekar et al. 2000, Bufe et al. 2002).

More recently it was shown that almost 20% of Gαi coupled GPCRs such as T2Rs cannot activate the human ortholog of Gα15, Gα16 (Kostenis 2001, Mody et al. 2000). In the human taste system *in vivo*, taste receptors for bitter, umami and sweet tastants couple most frequently to gustducin (Ueda et al. 2003, Wong, Gannon and Margolskee 1996). In gustducin knockout mice, intracellular calcium taste responses in individual taste cells are reduced by 70%. This demonstrated the importance of heterologous expression with taste receptors in cell models of taste (Caicedo et al. 2003). As such, following a study by Ueda et al., a chimeric Gα16 gustducin protein was created using Gα16 from human HL60 cells and 44 residues from the C terminus (including β6 sheet, α5 helix, and extreme C terminus domains) of rat αgustducin, termed Gα16gust44 (Ueda et al. 2003). This chimera was shown to couple effectively to T2R receptors in the HEK293 model and responses were comparable to those obtained from *in vitro* studies (Ueda et al. 2003). Cell-based assays have been used to study a range of taste receptors at the molecular level including hT1R2/hT1R3 sweet receptors (Toda, Okada and Misaka 2011), hT1R1/hT1R3 umami receptors (Nelson et al. 2002) and a range of T2R bitter receptors (Behrens et al. 2004, Kuhn et al. 2004, Narukawa et al. 2011, Brockhoff et al. 2007).

The assays have also been used for ligand identification of orphan bitter taste receptors (Meyerhof et al. 2010), study of the effects of polymorphisms on taste receptor activation (Bufe et al. 2005) and identification of taste receptor inhibitors (Pydi et al. 2014, Greene et al. 2011). However, to date, these studies have only focussed on HEK293 cell lines since they are easily transfected, have a fast doubling time and tend to have good translation of proteins (Thomas and Smart 2005). Since this is a kidney cell line, it may not provide a good representation of the human oral cavity. For this reason, the present study developed a novel cell-based assay for taste receptor activation using oral epithelial cell lines and a salivary layer to better mimic the oral environment and gain a better understanding of the role of saliva on taste reception.

1.9.2 Isolated Taste Cells

In addition to transfection of taste receptors in non-taste cells, live cell imaging has also been used on isolated taste cells from mice and rats. The procedure involves excising the tongue from the animal and enzymatic digestion to separate the lingual epithelium (Huang et al. 1999). Following this, individual taste cells are dissociated from the epithelium (Zhao, Lu and Herness 2002, Huang et al. 1999) or sections of the epithelium are stained with a calcium fluorophore and intracellular calcium responses measured (Chandrashekar et al. 2010, Caicedo et al. 2000a). The disassociation procedure was first described by Herness (1989) who used papain, a papaya fruit enzyme, with agitation to separate cells from the keratinised epithelium (Herness 1989).

Patch clamp techniques are also used on whole cells to measure the inward and outward currents in response to taste stimulation (Zhao et al. 2002). Patch clamp voltage measurements involve use of a patch pipette containing a solution of electrolytes and a recording electrode (Neher and Sakmann 1992). The cell is placed in a bath of fluid which mimics the extracellular environment (Spielman et al. 1989). The recording electrode is connected to an amplifier and contacts the cell membrane (Neher and Sakmann 1992). A reference electrode is placed in the bath and an electrical circuit is formed between the recording electrode, the cell and the reference electrode. The researcher can then observe changes in current as a function of taste activation (Neher and Sakmann 1992, Spielman et al. 1989).

The use of ex *vivo* taste cell imaging is beneficial since it allows for observation of mammalian taste cell responses at a molecular level. However, it is laborious and only

allows for measurement of single or small numbers of cells at a time. Additionally, the isolation procedure is not selective for taste cells, and as such, a proportion of non-gustatory epithelial cells will be present. Therefore, not all cells isolated are taste responsive and the assay is subject to high variability (Herness 1989). Furthermore, enzyme treatment during isolation of taste cells may affect responsiveness (Herness 1989). Additionally, when patch clamp techniques are used, if the stimulus is applied via the bath, it can access both the apical and basolateral cell domains resulting in responses which are not relevant to the *in vivo* environment (Akabas, Dodd and Al-Awqati 1988).

Taste cells are notoriously difficult to maintain in primary culture as they have a limited viability (Ruiz et al. 2001, Landin, Kim and Chaudhari 2005, Kishi et al. 2001). The most recent attempts to establish a primary taste cell culture by Ozdener et al. resulted in maintenance of rat foliate and circumvallate papillae cells for 3 months although cell morphology was changed after 1 month in culture (Ozdener et al. 2006). Furthermore, rat fungiform papillae could be passaged and maintained for 7 months in culture (Ozdener et al. 2011). These cells retained expression of key components of the taste signalling pathway, specifically gustducin and PLCβ2, as well as intracellular calcium responsiveness to taste stimulation (Ozdener et al. 2011). However, the cells did not ever reach full confluency and so the structure and organisation of taste tissue, which play a role in function were missing as the cells were not touching one another in culture (Ozdener et al. 2011). Additionally, over time, the cells underwent de-differentiation and exhibited alteration of chromosomes, which may affect gene expression in longer term cultures (Ozdener et al. 2011). As such, a primary taste cell culture is yet to be made available, but tongue epithelial cell lines do exist. In the present study a tongue epithelial cell line, SCC090, was characterised for expression of taste receptors and responsiveness to tastants in calcium assays which provided information for their use in taste research.

1.10 Factors affecting taste/olfaction in older adults

Table 1-2 (Imoscopi et al. 2012) Common Causes of Taste Loss in Older Adults

Physiological Changes in the oral cavity	Mucosae: ↓ thickness, dryness Salivary glands: ↓ acini, ↑ fibrous adipose tissues Tongue: ↓ density of taste buds
Diseases Oral	Caries, periodontal diseases, candidiasis, stomatitis, dental-alveolar infections, xerostomia, tumors, mechanical trauma
Systemic	CNS: stroke, mild cognitive impairment, Alzheimer's disease, Parkinson's disease, major depression Endocrine: diabetes mellitus types 1 and 2, hypothyroidism, hyperthyroidism Cancer: of the lung, breast, head and neck, esophagus, stomach Kidney: chronic renal failure Liver: acute and chronic liver diseases, cirrhosis Cardiovascular: hypertension Rheumatology: Sjoegren's syndrome Gastrointestinal tract: irritable bowel syndrome, gastroesophageal reflux disease, Crohn's disease Respiratory and viral diseases: chronic obstructive pulmonary disease, post-influenza conditions
Iatrogenic Drugs	Cardiovascular NSAID/corticosteroid Psychotropic Antibacterial Metabolic
Treatments	Chemotherapy/radiotherapy Surgery: middle ear surgery, tonsillectomy
Nutritional deficiencies	Malnutrition, zinc deficiency
Lifestyle	Smoking, alcohol consumption, poor hygiene of the oral cavity and dental prosthetic devices

1.10.1 Disease

There are several disease states known to impact upon taste ability in the elderly population, many of which are of increased prevalence in the aged. Recent studies have identified links between Alzheimer's disease, which is strongly linked with advanced age, and taste or smell ability. In fact, the loss of olfaction is thought to be one of the

earliest indicators of the disease (Mesholam et al. 1998). Previous studies have used neural imaging to investigate causes of olfactory decline in Alzheimer's patients showing that there are increased numbers of plaques in the areas of the brain where olfactory processing occurs (Figure 1-8). Cellular death and degeneration in these areas and neuronal tangling within the olfactory bulb have also been shown (Christen-Zaech et al. 2003) (Ohm and Braak 1987). Recently it was shown that Alzheimer's patients have increased detection threshold and reduced recognition for all basic tastes compared to age matched controls. However, no differences in responses were shown when using electro-gustometry (EGM) measurements (Ogawa et al. 2017). This implies that loss of taste in Alzheimer's disease is a result of the loss of sensory processing in the brain and not due to changes in the peripheral taste system.

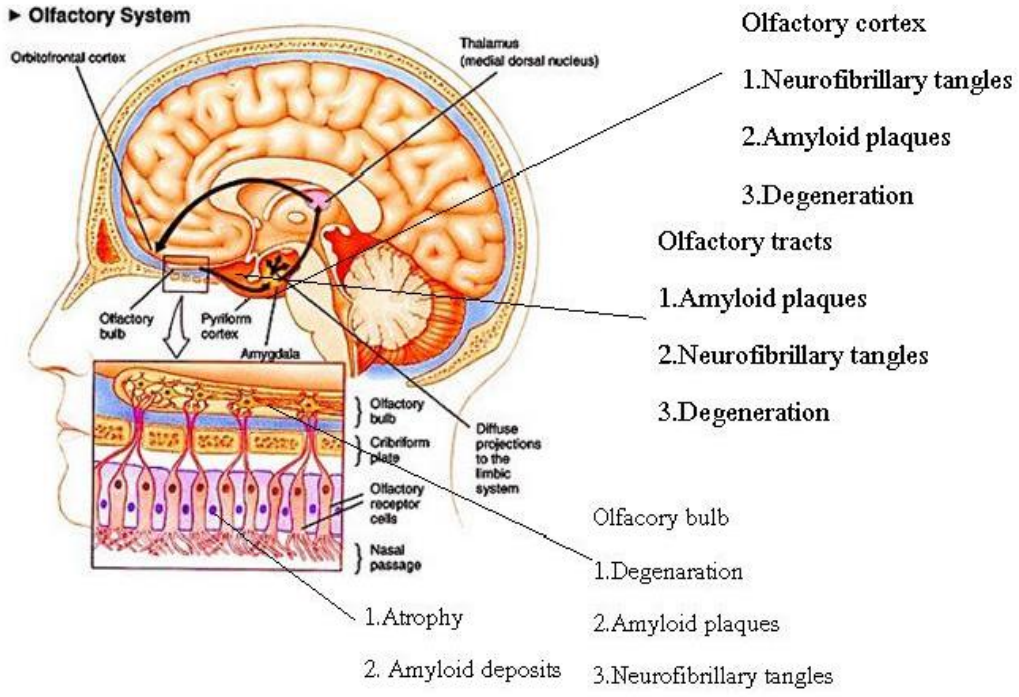


Figure 1-8 (Nicola-Antoniu 2011) Anatomical Changes of the Olfactory System Caused by Parkinson's Disease

Parkinson's disease is also an age related disorder which causes degeneration of olfactory senses, and therefore, as smell and taste are inextricably linked, may also lead to reduced taste sensitivity in the elderly (Doty 2012). The reason for this may be related to changes in neurotransmitter neurons, involved in carrying messages about odours, from nasal receptors to the hypothalamus in the brain. Dopamine and

noradrenaline are two neurotransmitters, required for correct olfactory function. Parkinson's patients have significant damage in the areas of the brain which send these neurotransmitters to the olfactory bulb (Huisman, Uylings and Hoogland 2008).

Cancer is another disease which can cause older adults to lose their sense of taste and smell. As well as head and neck radiation therapy, causing direct damage to taste buds and the olfactory system, studies have also shown a link between taste loss and various other cancers including lung, breast and renal. More specifically, taste alteration and a general metallic taste in the mouth have been observed in cancer patients (Ng et al. 2004). This has been related to altered zinc status in those who have had/are undergoing chemotherapy. This is because zinc deficiency is a known factor in taste decline, as well as altered taste bud cells, and can be caused by different types of cancer drugs (Zabernigg et al. 2010).

Upper respiratory diseases and infections are also common in the elderly who have weaker immune systems than younger people. Such infectious disease states can have a direct impact on taste and smell. This is due to blockages, damage and inflammation to the nasal cavity and damage to the airways where odorants are transported to receptor cells (Bromley 2000).

1.10.2 Effect of Hospitalisation and Institutionalisation

Several studies have found that older people living in the community are less susceptible to age related taste loss with lower recognition thresholds for sour taste than those who are hospitalised or living in residential care (Toffanello et al. 2013, Solemdal et al. 2014, Spitzer 1988). Higher thresholds for bitter taste in hospitalised older adults have also been identified (Solemdal et al. 2014). Additionally, a study testing sensitivity to suprathreshold concentrations of sweet, sour, salty and bitter tastants, showed decreased sensitivity for all tastes in residential living compared to community dwelling older adults (Ogawa et al. 2016). Older adults who have been hospitalised or live in residential care may have multiple co-morbidities impacting upon taste and smell sensation (Schiffman 1997). Long-term hospitalisation is often associated with poly-pharmacy and multiple disease states. On average, 67% of hospitalised older adults take five or more medications daily, upon discharge (Nobili et al. 2011). In residential homes 40% of older adults take more than four different medications every day while approximately 20% of those living in their own homes take the same number of

medications (Imoscopi et al. 2012). Additionally the oral health of older adults in residential care may be poor compared to those who are community dwelling (Simons, Kidd and Beighton 1999, Simons et al. 2001).

1.10.3 Medication

Table 1-3 (Imoscopi et al. 2012) Medications Commonly Linked with Taste Dysfunction in Older Adults

Class of Drug	Name(s) of drugs
Cardiovascular	Ace inhibitors: captopril Angiotensin blockers: losartan, valsartan, candesartan Calcium channel blockers: nifedipine, amlodipine, diltiazem
Diuretics	Amiloride, acetazolamide, spironolactone, furosemide
Beta-blockers	Labetalol, propranolol
Antiarrhythmic agents	Propafenone, amiodarone
Lipid-lowering agents	Atorvastatin, pravastatin
Anticoagulants	Clopidogrel
NSAIDs/Corticosteroids	Ibuprofen, diclofenac
Psychotropic agents	Tricyclic antidepressants, SSRI, mirtazapine, chlorpromazine, haloperidol, olanzapine, quetiapine, risperidone
Antibacterial agents	Penicillin, ciprofloxacin, clarithromycin, tetracyclines, antiviral drugs
Metabolic agents	Biguanide, thiamazole

Numerous medications have been linked to decline in taste sensitivity. Due to the fact that older people have a general decline in overall health, members of this age category are likely to be taking one or numerous medications on a daily basis (Schiffman 1993). Table 1-4 summarises medications which have an effect on taste function (Imoscopi et al. 2012). Over 47% of commonly prescribed medicines have had reported effects on taste, most commonly inducing a bitter taste in the mouth or reducing salivary flow which may reduce transport of taste molecules to the bud (Smith 1994). Medications found to reduce salivary flow include anti-depressants, beta blockers, diuretics, aspirin and diabetes drugs, all of which are commonly taken by older adults (Ichikawa et al. 2011). A study collecting information on medication use in a cohort of 300 adults, over 60 years old, of whom half were community dwelling and half were institutionalised,

found that the most common listed side effect was taste disturbance and the second most common was dry mouth (Deco et al. 2014). Since the study used listed side-effect information, however, this did not demonstrate the actual prevalence of taste loss/xerostomia in older adults taking certain medications. Not every individual taking a medicine will experience the listed side effects. Indeed, one limitation in general of studies investigating effects of medication on taste is that it is difficult to determine causal-effect relationships between individual medications and a side-effect. There is likely to be an interaction between different medicines in those taking more than one. Additionally, the side effect may not be directly linked to the medication since there may be co-morbidities such as the disease for which the medication is being taken.

1.10.4 Nutrition

Since elderly people have reduced taste, their food intake is also reduced, and so deficiency is common in this age category. The nutritional intake and status of elderly people could also be a factor in taste loss. For instance, a 2011 study by Kruse and Cambron (Kruse and Cambron 2011), found a tentative link between vitamin D deficiency and decreased sense of smell and taste, which were improved significantly by vitamin D supplementation. This was suggested to be due to vitamin D having a vital role in neuronal cell growth and function. Indeed, vitamin D deficiency may be highly prevalent in older adults, especially in those who become housebound due to poor mobility or ill health (Gloth et al. 1995). Since this study consisted of only 2 subjects it is clear that further work using a larger cohort is required. However, a possible link between nutrient deficiency and taste loss is suggested. A link between taste decline and dehydration has also been shown, especially in older adults (Ship and Fischer 1997). Dehydration may reduce salivary secretion and therefore impair transduction of taste molecules to receptor cells. Additionally, oral dryness can cause inflammation, cell damage and cell death in the oral cavity thus reducing taste sensitivity (Ship and Fischer 1997). Zinc deficiency is also related to taste loss in the elderly and has been shown to significantly decrease the ability of an individual to taste (Komai et al. 2000).

1.10.5 Oral Health

Poor oral health can significantly reduce taste sensitivity. Oral health is commonly poor in elderly individuals as they may lack ability due to diminished motor skills, cognition and access to provide good oral care (Petersen and Yamamoto 2005). A study by Solemdal et al. found a significant link between taste ability in patients with high

incidences of caries activity and bacteria levels in the mouth. Indeed, poor overall oral hygiene with thick layers of plaque on oral surfaces, tooth discolouration and bad breath were also linked with taste decline (Solemdal et al. 2012). Also, individuals who themselves complain of halitosis have been shown to have increased taste thresholds. This is likely because of strong oral cavity malodours masking pleasant tastes and odours (Nalcaci and Baran 2008). Furthermore, there is likely a direct impact of accumulation of bacteria on taste function since an undesirable plaque or film may form on the tongue which acts as a physical barrier to taste receptors (Solemdal et al. 2012, Feng et al. 2018).

1.10.6 Smoking

Smoking has an adverse effect on taste discrimination (Doty et al. 2017) and sensitivity to taste at suprathreshold level (Vennemann, Hummel and Berger 2008). However, many studies have failed to find a link between smoking and taste thresholds (McBurney and Moskat 1975, Vennemann et al. 2008, Konstantinidis et al. 2010). One study, using EMG to measure taste thresholds, showed increased electrical taste thresholds in smokers, compared to non-smokers (Coats 1974). However, EMG does not discriminate between taste modalities and essentially tests a sour taste response only. The pH of saliva in smokers is significantly lower than non-smokers and it is possible that a more acidic salivary pH may affect EMG taste responses (Parvinen 1984). Additionally, a recent study found increased sweet taste thresholds in smokers compared to non-smokers, however this was only tested in women (Pepino and Mennella 2007). Salivary secretory response to tastants was not shown to be impaired, in smokers compared to non-smokers (Khan, Javed and Ishaq 2010). Meanwhile, the adverse effect of smoking on the sense of smell, is widely accepted. Olfactory function is markedly reduced in smokers, compared to non-smokers and the effect is dependent on smoking duration and number of cigarettes smoked per day (Vennemann et al. 2008, Katotomichelakis et al. 2007, Frye, Schwartz and Doty 1990). Smoking is not directly related to old age, however there is some evidence to show that smoking intensity increases with age (Janson 1999). Also, smoking was more acceptable before the public health risks were identified, therefore older adults may have different beliefs around smoking and be more resistant to smoking cessation (Yong, Borland and Siahpush 2005, Parry, Thomson and Fowkes 2002).

1.10.7 Cognitive Decline

Cognitive decline can be associated with reduced taste perception (Steinbach et al. 2010) and olfaction (Peters et al. 2003). Prevalence of mild cognitive impairment in older adults ranges from 3-19%, depending on the population being studied (Ritchie 2004). Transmission of signals, following stimulation of the peripheral taste system, occur via cranial nerves VII, IX and X (Hamilton and Norgren 1984). The signal is then sent via the thalamus to the primary taste cortex, within the frontal operculum and insula where taste detection is processed (Rolls 1989, Small et al. 2003). Taste information is further processed, as perceived suprathreshold taste intensity, in the orbitofrontal cortex where taste pleasantness is detected, the amygdala which may process taste intensity and the cingulate gyrus, among other cerebral areas (Yamamoto et al. 1994, Small et al. 2003). Neurofibrillary tangles in the hippocampus, amygdala and entorhinal cortex, which lead to mild cognitive impairment, are generally present in the brain of older adults (Delacourte et al. 1999, Braak and Braak 1996). Also, atrophy in the hippocampus is associated with memory loss and age related cognitive decline (De Leon et al. 1997). MRI scanning of the hippocampus has shown hippocampal atrophy in a third of an older, >55 years, cohort (Golomb et al. 1993). Hippocampal damage can lead to impaired glucose metabolism, a sign of reduced synaptic activity, in the posterior cingulate. This has also been shown in patients, with an average age of 63.3 years, who experience mild cognitive impairment (Nestor et al. 2003). As such, the taste cortex is part of the region affected by age related deterioration occurring with cognitive decline (Steinbach et al. 2010). Patients with mild cognitive impairment have hyperactivation of the hippocampus and deactivation of the parietal lobe (Celone et al. 2006). Since parietal deactivation is linked with reduced recognition memory, individuals with mild cognitive impairment may subjectively believe their taste function is better than their actual level of sensation is (Celone et al. 2006).

1.10.8 Physiological Changes in the Taste System with Age

Counting taste papillae by eye is one method for determining the deterioration of taste bud numbers in ageing. Such studies in humans have found no difference in the visible papillae between older and younger subjects, although this is a fairly subjective measure (Mavi 1999). Additionally, using light microscopy to count taste buds from fungiform, circumvallate and foliate papillae in rhesus monkeys also failed to determine a difference in taste bud numbers as a function of ageing (Bradley, Stedman and Mistretta

1985). However, studies using excised tongue from mice together with immunohistochemical staining of taste cells have shown that while numbers of taste buds remain the same in ageing, the numbers of taste cells within each bud are significantly reduced (Shin et al. 2012). Further to this, the size of each taste bud is greatly reduced in an aged mouse compared to that of a young healthy mouse (Shin et al. 2012). These changes could be partly responsible for the observed losses in taste sensitivity with advancing age. Also, in human cadaver tongue tissue, reduced densities of taste bud and taste bud cells was shown within the older population compared to younger. This may demonstrate changes in cells which are parallel to changes in taste acuity (Shimizu 1997). Mouse studies have also been used to investigate cell turnover rates within taste buds of older mice compared to younger mice. There is a reduced turnover rate with new cells being replenished at a significantly slower rate in older mice despite numbers of type vi basal cells being similar in both age groups (Shin et al. 2012) (Fukunaga 2005). Also, double immunostaining for Ki67 - a nuclear protein required for cellular proliferation, in the circumvallate epithelium from mice has shown that the numbers of proliferating stem cells are reduced in older mice compared to younger (Feng, Huang and Wang 2013).

1.10.9 Genetic Factors

Polymorphisms in taste bud cell receptors may also be involved in prevalence pf changes to taste thresholds in the ageing population. Looking at bitter receptors specifically, there are five polymorphisms in TAS2 receptors which have been positively associated with advanced age (Campa et al. 2012). These polymorphisms have been suggested to influence food liking, as they cause functional changes in taste receptors. Three SNPs in the TAS2R16 gene (*rs6466849, rs860170* and *rs978739*), one in the TAS2R4 gene (*rs2233998*) and one in the TAS2R5 (*rs2227264*) were linked to longevity (Campa et al. 2012). Age does not appear to affect the prevalence of the PAV/AVI polymorphism of TAS2R38 gene. Relative numbers of PTC tasters:non-tasters has not been shown to be significantly different, between older and younger adults (Abraimov and Mirrakhimov 1979, Whissell-Buechy 1990).

Brain derived neurotrophic factor (BDNF), part of the neurotrophin family, is a growth factor involved in viability of neurons and plasticity of synapses in the central nervous system (CNS) (Lewin and Barde 1996). Recently, a role for BDNF has been described in the olfactory system where it may play a role in proliferation and growth of olfactory

neurons (Simpson et al. 2002). An SNP of the BDNF gene, val66met, was shown to be associated with greater decline in olfactory sensation in older adults (70-90years) (Hedner et al. 2010). Older adults, who are homozygous for the val form of the gene, display a greater loss of olfaction than met homozygotes and val/met heterozygotes (Hedner et al. 2010).

1.10.10 Changes in Saliva of the Elderly

Dehydration, medication and diseases common in elderly people are known to reduce the salivary flow rate. This can impact upon an individual's ability to taste, as saliva facilitates transport of tastants to the taste pore (Nagler and Hershkovich 2005b). Previous studies, looking at age related changes in salivary flow rate, have produced conflicting results. Some have found reduction in un-stimulated whole mouth salivary flow (Sawair et al. 2009, Fenoli-Palomares et al. 2004, Nagler and Hershkovich 2005b) as well as from the submandibular/sublingual glands (Yeh, Johnson and Dodds 1998). Others have shown reduction in stimulated whole salivary flow with advanced age (Smith et al. 2013, Toida et al. 2010) and some have found no differences in flow rate from the parotid gland (Percival, Challacombe and Marsh 1994, Ship and Fischer 1997) and in whole mouth saliva between older and younger adults (Salvolini et al. 2000, Bourdiol, Mioche and Monier 2004). Variable results from such studies may be due to different saliva collection techniques, variable subject numbers and different study designs. For example, circadian rhythms are known to affect saliva secretion, so inconsistency in collection times between studies could affect results (Dawes 1972).

In 2015, Affoo et al conducted a meta-analysis of 47 studies comparing salivary flow rate between older (>60 years) and younger (18-40 years) adults (Affoo et al. 2015). Data for different salivary condition, source, sex, health and collection method were collated and results analysed using meta-analysis software (Affoo et al. 2015). Overall, the review demonstrated that un-stimulated and stimulated salivary flow rates were lower in older adults compared to younger adults. The greatest age related reduction in salivary flow rate was in un-stimulated salivary flow (Affoo et al. 2015). There was no apparent effect of age on parotid salivary flow rate but there was an effect on submandibular/sublingual flow. Since stimulated whole mouth saliva is over fifty-percent parotid saliva, this explains the greater age related reduction in un-stimulated salivary flow compared to stimulated (Affoo et al. 2015). Changes in submandibular

gland and minor labial gland tissue have been shown in ageing with a reduction in acinar volume and secretory tissue coinciding with an increase in fatty and fibrous tissue (Scott 1977, Drummond and Chisholm 1984). Preserved parotid saliva secretion, in advanced age, may be due to salivary gland secretory reserve cells. Healthy adults may have more secretory cells than needed for normal salivary flow. As such, age related deterioration in secretory tissue does not necessarily affect function (Ghezzi and Ship 2003, Scott 1977).

Saliva also protects taste cells and provides hydration, growth factors and antibacterial action, to keep taste buds healthy (Mese and Matsuo 2007). Salivary composition is altered with advanced age with greater concentration of salivary proteins and electrolytes due to reduced flow of the watery part of saliva (Ship and Fischer 1997). This affects the way saliva can lubricate and protect oral surfaces including the taste buds. Additionally, protective factors such as salivary mucins, immunoglobulins and enzymes have been shown to be reduced in saliva from older adults compared to that of younger adults (Vissink, Spijkervet and van Nieuw Amerongen 1997, Denny et al. 1991). A lower concentration of histatins - salivary proteins which play a role in oral anti-bacterial defence has also been demonstrated in whole saliva from older adults (Johnson, Yeh and Dodds 2000). Further, expression of the protective surface-bound mucin, MUC1, is reduced on oral epithelial surfaces of older adults compared to younger (Chang et al. 2011). MUC1 facilitates muco-adhesion of the salivary pellicle in the mouth and reduced MUC1 expression was associated with poor protection of the oral mucosa and reduced lubrication due to poor mucin binding (Chang et al. 2011).

To date, few studies have investigated the effect of ageing on salivary viscoelasticity. Zussman et al. used an elongational viscometer to measure relaxation times in saliva from younger (n=11) and older adults (n=22) to show that there was an increase in median relaxation time of saliva from the older group although this was not statistically significant (Zussman et al. 2007). The use of median values was reported due to high inter-individual variation and as such the use of a small participant group may have impacted upon the results. No studies, to date, have looked at the effect of ageing on taste stimulated salivary viscoelasticity. Since saliva is secreted as a response to taste, smell and trigeminal stimulation, the physical properties of the secretion may vary depending on the magnitude of the response. Further, the viscoelasticity of stimulated saliva is dependent on stimulus type and concentration (Stokes and Davies 2007) as the

proportion that each salivary gland is contributing to the whole salivary flow varies. Parotid saliva has little viscoelasticity while submandibular/sublingual saliva is mucin rich and therefore highly visco-elastic (Van der Reijden et al. 1993). There are few studies which have looked at the effect of ageing on stimulated salivary viscosity. Briedis et al. used cone and plate rheometry and showed no significant difference in viscosity of chewing stimulated saliva between older (>60 years) and younger adults (21-30 years) (Briedis, Moutrie and Balmer 1980). This is perhaps not surprising given the fact that chew stimulated saliva is composed mainly of parotid saliva which contains little to no mucin (Vijay et al. 2015). Parotid salivary flow is largely retained in older adults (Affoo et al. 2015) suggesting retained function of the parotid gland and therefore a low viscosity of chewing stimulated saliva, regardless of age, would be expected. The problem of low flow rate in older adults means that traditional methods for measuring salivary viscosity may be un-suitable. Indeed, Briedis et al. reported that there were multiple saliva samples which could not be tested for viscosity due to insufficient volume (Briedis et al. 1980).

Saliva may also contain taste binding proteins, which facilitate taste transduction to the receptor (see 1.5.3 Salivary Growth Factors and Proteins Required for Taste). As such, reduced salivary flow and changes in salivary composition may result in changes in the levels of these proteins thus contributing to taste loss.

1.11 Aims and Objectives of the PhD

1.11.1 Aims

Age related loss of taste and smell sensation is evident and has been studied extensively using a variety of testing methods. It has been previously demonstrated that saliva has an important role in taste but only some mechanisms for this been elucidated thus far. Furthermore, the role of saliva specifically in taste loss of the ageing population is not well studied. Saliva has a complex rheology with mucins which impart high viscoelasticity and facilitate muco-adhesion. Therefore, the focus of the study was to investigate the role of rheology of saliva on age related taste loss. Also, measuring of salivary response to taste stimulation would provide an objective measure for taste which is easy to replicate, inexpensive and non-invasive, giving valuable insight into taste loss amongst the older population. *In vitro* models of taste have also been used to obtain further information about taste receptor activation on a molecular level, however, a simple model which closely mimics the oral environment has not yet been established. With that in mind and to enable better understanding of the role of physical properties of salivary in age related taste loss, the present study has the following aims:

1) To investigate the effect of ageing on subjective (reported perceptions) and objective (salivary responses) measures of taste, olfaction and chemo-sensation (TRP receptor stimulation).
2) To investigate changes, with age, in salivary physical properties, including viscoelasticity and viscosity and salivary mucins, which impart extensional rheology to saliva and thus, may impact upon diffusion of tastants and muco-adhesion to the oral epithelium.
3) Develop and utilise an *in vitro* model to enable study of age related taste loss using taste receptive cultured oral epithelial cells. The model will allow in-depth analysis of the effect of age related changes in saliva on taste at the receptor level where salivary-taste receptor interactions are likely to occur.

1.11.2 Objectives

In line with the above aims, the objectives below were addressed to achieve the goals of the project and attain the overall aim of discovering the role for physical properties of saliva in taste and the effects of changes in saliva in older adults:

- Conduct a human taste test study and compare taste, TRP and odour responses from younger (aged 18-30 years) to older (aged 60+ years) adults using a subjective measure in the form of a labelled scale and an objective measure in the form of salivary flow rate and altered physical properties in response to stimuli
- Measuring pH of un-stimulated and stimulated saliva to observe salivary pH changes in ageing and following TRP, taste and odour stimulation
- Rheological measurements such as viscoelasticity, in the form of spinnbarkeit, and viscosity taken to assess the physical changes occurring in saliva using different forms of stimulation
- Protein analysis of saliva samples to characterise the composition of saliva from older and younger adults, including analysis of salivary mucin composition, MUC7 and MUC5B, using western blotting, SDS PAGE gel glycoprotein staining and lectin blots to semi-quantify mucin levels and glycosylation of salivary mucin in both age groups
- Develop an *in vitro* model for muco-adhesion to quantify the mucin binding capability to the oral mucosa of saliva from older subjects compared to younger
- Testing of multiple oral and non-oral epithelial cell lines for endogenous expression of taste receptors and for efficiency of transfection
- Development of a cell-based model for the study of taste loss using epithelial cells which will be transfected with a TAS2R38 taste receptor and intracellular calcium responses to TAS2R38 agonists, representative of bitter taste, measured in the presence of a salivary layer

2 Methods and Materials

2.1 Study Group

31 younger (27 female, 4 males, 18-30 years old, mean age 24.3 +/-0.4 years old) and 25 older (19 female, 6 males, 60+ years old, mean age 72.4 +/-1.8 years old) healthy participants took part in this study. Smokers, pregnant or breast-feeding women and those with swallowing difficulties were excluded. This study was carried out in accordance with the recommendations of King's College London Guidelines on Good Practice in Academic Research. The protocol was approved by the King's College London Biomedical Sciences, Dentistry, Medicine and Natural & Mathematical Sciences research ethics committee (BDM RESC), application reference: BDM/12/13-130. All subjects gave written informed consent in accordance with the Helsinki Declaration.

2.2 Saliva Collection

Taste compounds and TRP agonists used were food grade (Sigma Aldrich, Poole, Dorset, UK). Volunteers were asked to donate taste stimulated and unstimulated whole mouth saliva (UWMS) samples. Collections were carried out for 2 minutes for UWMS and for stimulated (SWMS) following a 1-minute rinse with 1ml solution (0.05M monosodium glutamate, 0.05 M caffeine, 0.05M menthol, 0.005M capsaicin, all dissolved in 1% propylene glycol (PG) and spring water). Concentrations were chosen because preliminary testing revealed that these concentrations elicited a salivary flow rate response greater than that elicited by water alone. A water rinse control was used consisting of 1% PG in spring water. The stimulating solution was expectorated before collection of saliva. For odour stimulation, a 2-minute saliva collection was carried out after 1-minute smelling menthol crystals. Tubes were pre-weighed used and weighed again after collection of saliva to allow calculation of flow rates (g/min). pH of saliva was determined using a portable pH meter (accuracy ± 0.02 pH, Omega Engineering Ltd. Manchester, UK), only for samples with a volume great enough for the pH meter probe to be completely submerged, approximately 1ml. Samples were stored on ice immediately after collection, aliquoted to minimize freeze thaw cycles and stored at -80°C.

2.3 Subjective Taste Perceptions

Participants were asked to rate the intensity of all administered tastants (including water rinse control), TRP and odour compounds using a labelled scale from no sensation to the strongest imaginable sensation (Appendix 1, adapted from (Hayes, Allen and Bennett 2013). PTC taste test strips were prepared as described by Zhao et al. (2003) by soaking 2x2cm squares of filter paper in 50mM PTC solution (dissolved in spring water, heated gently) and leaving to air dry (Zhao et al. 2003b). Participants were asked to rate the intensity of the PTC strip after placing the strip on the tongue for 3 seconds then removing. The same labelled scale was used for PTC testing and the other stimulants.

2.4 Beliefs About Taste and Smell Loss – Qualitative Study

To assess beliefs around taste and smell loss a validated questionnaire was created (Appendix 2). 8 questions about taste and smell loss and eating behaviours were included as well as 9 questions with no relevance to control for bias. Questions about demographics were derived from the 2011 UK national census (Office for National Statistics (ONS), 2011). Questions about daily medication use were also included. Participants were first asked if they could remember a significant event (the London 2012 Olympics) in the past 5 years to encourage accurate recall and then to relate all answers to before and after that event. All participants were asked to complete the questionnaire and return it after taking part in the taste test study. In the 18-30 years old group, 8 individuals returned the questionnaire. In the 60+ group 11 individuals returned the questionnaire. Data was collated and expressed as percentage respondents within each group.

2.5 Rheological Analysis

2.5.1 Extensional Rheology (ER)

The NevaMeter Extensional Rheometer (Ishikawa Iron Works, Japan) was used to carry out measurements of viscoelasticity (Spinnbarkeit) on samples. A 100µl sample was loaded onto the machine. Measurements were conducted in triplicate and an average taken. The Spinnbarkeit was determined as the point at which electrical conductivity of the saliva was broken after being subjected to constant stretching at a rate of 5 mm/s.

2.5.1 Differential Dynamic Microscopy (DDM)

UWMS samples were thawed on ice and mixed with 1 µm fluorescent beads coated with polyethylene glycol (PEG) and sealed under a glass cover slip on a microscope slide using UV cured glue. Slides were imaged in bright field using a 40X magnification on a DM IRBE inverted microscope (Leica Microsystems, Bannockburn, IL) on a vibration-dampened table. An area scan high-speed monochromatic digital video camera (model No. A602f-2; Basler, Ahrensburg, Germany) was used for image and video acquisition, recording a 10 second video with a resolution of 1200x1200 pixel (px) and 160 fps. Images were taken from 3 distinct regions of each sample and an average taken to control for heterogeneity of the WMS samples. Images were acquired at room temperature (average 25°C). Diffusion coefficient (D) of the suspended fluorescent beads of radius (r) was measured using DDM methods previously described (Cerbino and Trappe 2008). From the diffusion coefficient (D) it was possible to extract the viscosity of the sample using the Stokes-Einstein equation: viscosity (Pa.s) = Kb x T/(6 x π x D x r), where Kb is the boltzmann constant, T is the temperature and r is the radius of the particles.

2.6 Analysis of Proteins

2.6.1 Total Protein Concentration

The Bicinchoninic acid assay (BCA assay) was used to determine the total protein concentration of samples. Bovine Serum Albumin was used as a standard, 2mg/ml-0.025mg/ml. Duplicate samples of BSA at 2, 1.5, 1, 0,5, 0.25, 0.125, 0.025mg/ml, a water control, and samples were added to a 96-well plate and absorbance at 540nm was measured using the iMark Microplate Absorbance Reader (BIORAD, UK). Protein concentrations of samples were calculated with a standard curve created by plotting on a linear graph, the standard concentration (mg/ml) against absorbance (nm). A linear equation was generated from the graph and used to calculate sample protein amounts from absorbance readings.

2.6.2 Gel Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to analyse protein composition of samples. Samples were prepared in NuPAGE lithium dodecyl sulfate sample buffer (LDS) sample buffer (25%) (Invitrogen, Calsbad, USA) under reducing conditions (50mM dithiothreitol (DTT)). Samples were boiled for 3

minutes at 100°C and 50µg total protein loaded onto the gel (Xcell electrophoresis unit (Invitrogen, Calsbad, USA)) with MES SDS running buffer (50 mM MES, 50 mM Tris Base, 0.1% SDS, 1 mM EDTA, pH 7.3 (Invitrogen, Calsbad, USA)) at 200volts constant for 32 minutes.

2.6.3 Periodic Acid Schiff (PAS) Glycoprotein Stain

High molecular weight glycoproteins (MUC5B and MUC7) were visualized using the PAS stain. Gels were fixed using 25% methanol, 10% acetic acid solution for 1 hour. Following three, five-minute washes in Ultra High Quality (UHQ) water, gels were oxidised using 2% periodic acid solution (Sigma Aldrich). After another 2 x UHQ water washes, Schiff's reagent was added, and gels were left to stain for 45 minutes in dark conditions. Water was used to de-stain and gels were imaged using Syngene Gene Genius Bio Imaging System (Cambridge, UK).

2.6.4 Western Blotting

To detect antibodies for specific proteins (immunoblot) gels were transferred, using western blotting, onto nitrocellulose membranes via electrophoresis. The gel was sandwiched between a nitrocellulose membrane and filter paper then another filter paper was placed on top of the membrane. Sponges soaked in transfer buffer (NuPage Transfer Buffer (Invitrogen, Carlsbad, USA), 10% Methanol, UHQ water) were placed beneath and on top of the gel sandwich. The gel sandwich was placed into a blot module so that the nitrocellulose membrane was towards the anode in an Xcell vertical electrophoresis unit (Invitrogen, Carlsbad, USA). Transfer was for 60 minutes at 30 volts constant and 200 amps.

Following transfer, the gel was discarded, and membranes placed transfer side up in clean plastic trays. Membranes were blocked for 1 hour in 2%-5% (w/v) non-fat milk powder (NFM) (Marvell, Premier Foods, London, UK) dissolved in TBS-T solution (Tris-Buffered Saline, 20mM Tris, 0.9% NaCl and 1% Tween 20, pH 7.6). After washing membranes twice for 5 minutes each in TBS-T, they were incubated in primary antibody solution for 1 hour at room temperature or overnight at 4°C. Blots were washed 6 times for 2.5 minutes in TBS-T and then incubated in secondary antibody for 1 hour at room temperature. An antibody for beta actin was used as a loading control for cell lysates while amylase was used as a loading control for saliva samples.

After washing again, antibody signal was visualised using chemiluminescent substrate Luminata Crescendo Western HRP (Merck Millipore, Watford, UK). The blots were exposed to x-ray film (Photon Imaging Systems Ltd, Swindon, UK) for 30 seconds-5 minutes (dependent on signal) to detect signal. The JPI automatic x-ray film processor model JP-33 (Jungwon Precision Industries Ltd. Seoul, Korea) was used to develop films with RG rapid x-ray developing solution and x-ray fixer (Champion Photochemistry, Kuala Lumpur, Malaysia). Films were processed for 90 seconds at 37°c followed by fixative for 3 minutes and drying at 50°c. All the exposure and developing steps were conducted in dark conditions.

Table 2-1 Antibodies used for immuno-blotting

Primary Antibody	Manufacturer	Dilution	Host Species	Secondary Antibody	Dilution of Secondary Antibody	Dilutant
Human Carbonic Anhydrase VI	Santa Cruz Biotechnology, Dallas, Texas, USA	1:500 dilution	Goat	Rabbit-anti goat (Sigma, Dorset, UK)	1:10,000	2% NFM TBST
Human Cystatin S	Santa Cruz Biotechnology, Dallas, Texas, USA	1:1000	Goat	Rabbit-anti goat (Sigma, Dorset, UK)	1:10,000	2% NFM TBST
Human α-amylase	Santa Cruz Biotechnology, Dallas, Texas, USA	1:200	Goat	Rabbit-anti goat (Sigma, Dorset, UK)	1:10,000	2% NFM TBST
Polyclonal MUC1	Genetex International Corporation, Irvine, CA 92606 USA	1:1000	Rabbit	Swine-anti-rabbit igG (Dako, Santa Clara, CA, USA)	1:3000	TBST
EU-MUC5Bb	Novus Biologicals, Abingdon, UK	1:500	Mouse	Goat-anti-mouse igG (Sigma, Dorset, UK)	1:10,000	TBST
EU-MUC7a	Novus Biologicals, Abingdon, UK	1:500	Mouse	Goat-anti-mouse igG (Sigma, Dorset, UK)	1:10,000	5% Ovalbumin TBST
hTAS2R38	Abcam, Cambridge, UK	1:500-1:3000	Rabbit	Swine-anti-rabbit igG (Dako, Santa Clara, CA, USA)	1:3000	5% NFM TBST
beta Actin Loading Control Monoclonal Antibody	Thermo Fisher Scientific (Life Technologies), Paisley, UK	1:5000	Mouse	Goat-anti-mouse igG (Sigma, Dorset, UK)	1:10,000	5% NFM TBST
Monoclonal [M2] to DDDDK tag - (Equivalent to FLAG antibodies)	Abcam, Cambridge, UK	1:500	Mouse	N/A (HRP conjugated primary)	N/A	5% NFM TBST

Anti-HSV Tag (Clone HSV 78)	Sigma, Dorset, UK	1:500	Mouse	Goat-anti-mouse igG (Sigma, Dorset, UK)	1:10,000	5% NFM TBST

2.6.5 Detection of Sialic Acid

To detect sialic acid residues in saliva, UWMS samples were electrophoresed by SDS-PAGE and transferred to nitrocellulose as described above. After transfer, membranes were washed in TBS-T (Tris-Buffered Saline, 20mM Tris, 0.9% NaCl and 1% Tween 20, pH 7.6) for 30 minutes at room temperature. Sambucus nigra agglutinin (SNA, 0.05 µg/ml) biotinylated lectin, was used to detect α-2, 6 linked sialic acid, and Maackia amurensis leukoagglutinin II (MAL II, 0.4 µg/ml) biotinylated lectin, used for α-2, 3 linked sialic acid (Vector Laboratories, CA, USA), diluted in TBS-T and incubated at room temperature for 60 min. Following washing in TBS-T, 6 x 2.5 minutes, signal from bound lectins was detected using horseradish peroxidase linked streptavidin (Vector Laboratories). After washing again in TBS-T, lectin signal was visualised using chemiluminescent substrate Luminata Crescendo Western HRP as described above (Merck Millipore, Watford, UK).

2.6.6 Semi-Quantification of Immuno-Blots

Densitometry was conducted to semi-quantify band intensities of western blots. Western blot films were scanned, and images were imported into Image J version 1.46 (NIH, MD, USA) for semi-quantification of the band pixel intensity.

2.6.7 Immuno-cytochemistry

For detection of TAS2R38 protein expression in transfected and un-transfected TR146 cells, fluorescent confocal microscopy was used. Cells were seeded at 2.5 x 10^5 in coverslip bottom dishes and grown overnight at 37°C, 5% CO2, or transfected 24 hours prior to conducting immunocytochemistry. Media was removed, and cells were washed with PBS before fixing for 10 mins with 100 % MeOH at room temperature. Cells were washed with PBS three times for 2 minutes each wash and incubated with PBS + 1 % BSA for 45 mins to block non-specific protein binding. Following this, PBS BSA was removed and replaced with PBS 0.1 % BSA for 15 mins incubation. After another wash step, cells were incubated for 45 minutes in primary antibody diluted in PBS + 0.1 % BSA (TAS2R38 antibody diluted 1:3500). Three further PBS/0.1% BSA washes were

performed, and secondary antibody added (goat anti rabbit fluorescein isothiocyanate (FITC) conjugated igG 1:500 dilution, Abcam, Cambridge, UK) diluted in PBS 0.1% BSA, for 45 minutes incubation in dark conditions. Cells were washed in PBS three times and counter stained with Hoechst 33342 (Promokine, Heidelberg, Germany) to allow visualisation of the cell nuclei. Hoechst is a cell-permeable stain which binds preferentially to adenine-thymine (A-T) regions of DNA and fluoresces blue at wavelengths of 460 to 490 nm. A staining solution was prepared by adding 25ul Hoechst 33342 to 500ul PBS. Cells were incubated with staining solution for 15 minutes at room temperature in dark conditions. Cells were washed again in PBS and then 1ml PBS added to each dish before visualisation of fluorescence using AR1 confocal microscope (Nikon, UK) at 40X magnification, with excitation wavelengths of 488nm (FitC) and 404nm (Hoechst 33342). Fluorescence of a region of interest was quantified using NIS Elements software (Nikon, UK). Experiments were performed in triplicate and an average was calculated.

2.7 Cell Culture

Experiments were carried out using the TR146 buccal epithelial carcinoma cell line stably transfected to express human MUC1 (a kind gift from Professor Martine Morzel, INRA, Centre des Sciences du Goût et de l'Alimentation, Dijon, France) and SCC090 squamous cell carcinoma cell line from the base of the tongue. TR146 cells were used as a control in optimisation experiments. TR146/MUC1 and TR146 cells were cultured in DMEM/F12 medium (Gibco), supplemented with 15% foetal bovine serum (FBS) and 100 units/ml penicillin, 100 μg/ml streptomycin in T175 flasks (Life Technologies, Carlsbad, CA, USA). SCC090 cells were cultured in Eagle's Minimum Essential Medium (EMEM) supplemented with non-essential amino acids (L-Alanine 0.89 g/L, L-Asparagine 1.5 g/L, L-Glutamic Acid 1.47 g/L, Glycine 0.75 g/L, L-Proline 1.15 g/L, L-Serine 1.05g/L), 10% FBS and 1% gentamycin (all Sigma, Dorset, UK). TR146 cells were sub-passaged every 3-4 days with medium changes every 2 days. SCC090 cells were sub-passaged every 5 days with medium changes every 2 days. Cells were incubated at 37 °C, 5% CO2.

For the assays, TR146 cells were seeded at a density of 5×10^5 cells/ml and SCC090 were seeded at a density of $1x10^6$/ml, incubated for 24 hours until >80% confluency was achieved.

2.7.1 Cell freezing and thawing

Trypsinised cells were re-suspended in cell culture media and centrifuged at 4°C at 300 x g for 5 min. The supernatant was discarded and the cell pellet (minimum 2 x 10^6 cells) was re-suspended in 1 mL of freezing medium (90% (v/v) FBS and 10% (v/v) DMSO) and transferred to a cryovial. Cryovials were stored at 80°C in an isopropanol bath (Mr Frosty, Thermo Scientific) to enable freezing at a cooling rate of -1°C per minute. Following 7 days storage, vials were transferred to a liquid nitrogen tank (200°C) for long-term storage.

To re-culture cells from frozen stocks, cells were thawed rapidly in a 37°C water bath, and resuspended in 10 mL of culture medium before centrifuging at 4°C at 300 x g for 5 min. The supernatant was discarded, and the cell pellet was re-suspended in 25ml of fresh media in a T175 tissue culture flask (TR146) or 15ml media in a T75 flask (SCC090). Flasks were incubated at 37°C, 5% CO2.

2.7.2 Cell Counting

In order to ensure equal cell seeding for experimental work, cells were first trypsinised using 0.5 ml/10cm2 pre-warmed trypsin/EDTA (for TR146 cells 0.05%, for SCC090 cells 0.25%) (Gibco, Paisley, UK) for 3-15 minutes at 37°C. Cells were re-suspended 1:2 with cell culture medium. 100 µl of cell suspension was mixed 1:1 with trypan blue (Sigma-Aldrich, Cambridge, UK). The mixture was then pipetted into a haemocytometer (Hawksley, Sussex, UK), so that the counting grid was fully covered. Viable cells in the 4 outer squares were counted and the following calculation was used to determine the cell number per ml of medium:

(counted cells ÷ 4) x 2 (dilution factor) x 10^4 = cells/ml in cell suspension

2.8 Mucin Muco-Adhesion Assay

For the assay, TR146 cells were seeded at a density of 5×10^5 cells/ml and SCC090 were seeded at a density of $1x10^6$/ml, both in 12-well culture plates, incubated for 24 hours until >80% confluency was achieved. A layer of saliva was added onto cells by incubating sub-cultures for 2 hours with saliva diluted 1:1 into growth medium, as previously described (Ployon et al. 2016). Saliva samples collected from older and younger adults (described above, 2.2 Saliva Collection) were clarified by centrifugation at 2000G for 10 minutes at 4°C before depositing onto the cells. Following the

incubation period, samples were washed twice with PBS to clear any non-adsorbed salivary mucin. Cells were lysed using a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich, Dorset, UK) for 2 minutes on ice before scraping using a cell scraper. Lysates were transferred to Eppendorf tubes and incubated for 20 minutes on ice. The lysates were centrifuged at 13,300G for 10 minutes at 4°C to eliminate cell debris. The supernatant was collected and used for western blotting of mucins and lectin blotting of sialic acid residues. Lysates were stored at -20°C between analysis and kept on ice during experimental work to prevent degradation by proteases.

2.9 Transfection and mRNA Analysis

2.9.1 Purification of plasmid DNA

Bacterial media used:

Luria Bertani medium (LB): 10 g/L Tryptone (Oxoid, Cambridge, UK); 5 g/L Yeast extract (Oxoid, Cambridge, UK); 10 g/L NaCl (Sigma-Aldrich, Dorset, UK).

Luria Bertani Agar (LBA): 10 g/L Tryptone (Oxoid, Cambridge, UK); 5 g/L Yeast extract (Oxoid, Cambridge, UK); 10 g/L NaCl (Sigma-Aldrich, Dorset, UK); 15 g/L Agar Bacteriological (Oxoid, Cambridge, UK).

SOC medium: 20 g/L Tryptone (Oxoid, Cambridge, UK); 5 g/L Yeast extract (Oxoid, Cambridge, UK); 0.5 g/L NaCl; 4.8 g/L MgSO4 (Sigma-Aldrich, Dorset, UK).

hTAS2R38 PAV plasmid DNA was a kind gift from Professor Maik Behrens, Leibniz-Institute for Food Systems Biology at the Technical University of Munich. The Gα16Gust44 chimeric construct was a gift from Professor Takashi Ueda, Division of Cellular Dynamics, National Institute for Basic Biology (NIBB), Okazaki, Japan. Preparation of this construct is described in the paper "Functional interaction between T2R taste receptors and G-protein α subunits expressed in taste receptor cells" (Ueda et al. 2003). Plasmid maps are shown in Appendix 3.

Plasmid DNA was purified by transforming XL1-blue competent *E. coli* (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Briefly, 1ng plasmid DNA was added to 100µl competent cells with 1.7 µl of β-mercaptoethanol and

incubated on ice for 30 minutes. The cells were heat shocked in a 42°C water bath for 45 seconds before rapidly chilling on ice for 2 minutes. The mixture was diluted with 0.9 ml of preheated SOC medium and incubated at 37°C for 1 hour with shaking at 225–250 rpm. 50-100µl of cell mixture was plated onto LBA plates supplemented with carbenicillin 50 µg/ml. Plates were incubated overnight at 30°C and individual colonies selected for 5 ml cultures, grown overnight in LB supplemented with carbenicillin 50 µg/ml. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (QIAGEN, West Sussex, UK) following the manufacturer's instructions. Cells were harvested by centrifugation and the pellet was re-suspended in a buffer of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, and 100 µg/ml RNase A. The lysis buffer is composed of 200 mM sodium hydroxide (NaOH) and 1% Sodium Dodecyl Sulphate (SDS). After addition of Potassium Acetate (CH3COOK), to neutralise, lysates were cleared by centrifugation. Clarified lysates were applied to a silica membrane, which selectively absorb DNA. The membrane was washed twice with ethanol to remove RNA, proteins and salts and the plasmid DNA was eluted in 50 µl of deionised water. Mini-prep DNA samples were used in subsequent analysis steps, namely restriction digest and agarose gel, to confirm presence of the correct size DNA fragment.

Subsequently, DNA was purified for use in transfections using GeneJET Endo-Free Plasmid Maxiprep Kit (Thermo Fisher Scientific (Life Technologies), Paisley, UK). A 5ml starter culture of LB broth was grown at 30°C, overnight, from individual colonies of transformed *E. coli* picked from LBA plates prepared as described above. This was diluted 1/1000 with LB broth and grown overnight at 30°C with shaking at 225rpm. Cells were harvested by centrifugation and the pellet re-suspended in a buffer of 50 mM Tris-HCl pH 8.0, 10 mM EDTA, and 100 µg/ml RNase A. Neutralisation buffer was added, followed by the Endotoxin Binding Reagent, and the lysate was filtered before being applied to a silica membrane. Following ethanol washes (x2) DNA was eluted in 500µl deionised water. The presence of the correctly sized DNA fragment was confirmed using restriction digestion with agarose gel electrophoretic analysis, before purified DNA was used in transfection experiments.

2.9.2 Primer Design and Reconstitution

To minimise amplification of genomic DNA, primer pairs were designed, where possible, so that at least one primer in a pair would span an exon-exon boundary to prevent the amplification of genomic DNA. To design primer sets, the Roche universal

probe library assay design centre software was used (https://lifescience.roche.com/). A list of potential primer sets for each target was generated by the software and all primers had an optimal annealing temperature of ≈ 60˚C. Primer sets were assessed for specificity to the target gene using the NCBI nucleotide BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 2-2 Oligonucleotides used

Oligo Name	5'-3' sequence	Expected Product Size (Base Pairs)	Melting Temperature (°C)
Gα16Gust44 forward	CCT GGT TCA AAA GCA CAT CCG	254	68.6
Gα16Gust44 reverse	TTG GGT GTC AGT AGC ACA GGT		64.6
YWHAZ forward	ACT TTT GGT ACA TTG TGG CTT CAA	94	65.3
YWHAZ reverse	CCG CCA GGA CAA ACC AGT AT		66.0
TAS2R7 forward	GGA TTC TAC TGG GGT GCG TGG T	260	70.8
TAS2R7 reverse	ATA GTC CGC TTA CGT CGA GTC AC		65.4
TAS2R43 forward	ATC TGG GCA GTG ATC AAC CA	156	65.6
TAS2R43 reverse	TAG CAA AGG CCC CAA CAA CA		67.5
TAS1R1 forward	AGC ACA GAA AAA TGG CTC CG	206	59.12
TAS1R1 reverse	GGC ACA ATA GAA AAC GCC GA		59.20
TAS1R3 forward	ACG TTC TCT GTC TAC GCA GC	439	60.11
TAS1R3 reverse	ATG TCG TCT GGG TTT TGC C		60.11
TAS1R2 forward	GCA CCT TCC TCA ACC ACA CT	532	60.18
TAS1R2 reverse	GTG GCC AGC ATG CCA ATT AC		60.18
TAS2R38 forward	AGG CCC ACA TTA AAG CCC TC	204	60.03
TAS2R38 reverse	CAG CTC TCC TCA ACT TGG CA		59.96
TAS2R10 forward	GTG TAG TGG AAG GCA TCT TCA	296	61.7

TAS2R10 reverse	GCT GGT GGC AAA CCA CAT AC		65.3
TAS2R38/TAG_HSV Fusion (for sequencing) forward	AGC AGC CTG AAG	N/A	31.0
TAS2R38/TAG_HSV Fusion (for sequencing) reverse	AAA GCC GGC TGA TGC TGA		67.2
Oligo dT	TTT TTT TTT TTT TTT TTT	N/A	
HSV Epitope D forward	TTG CCT TCA TCT CTG TGC CC	260	60.32
HSV Epitope D reverse	TTA ATC TTC CGG GTC TTC AGG AG		62.2
GNAT 3 (human gustducin) forward	TAC CCT GGA AGA TGG TGG CA	642	63.7
GNAT 3 (human gustducin) reverse	GCA CAG GTC ATG TGG GAA TAA		60.8

2.9.3 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was conducted by mixing 10ng of plasmid DNA, in 50 µl total of PCR reaction mixture comprised of: dNTPs (deoxyribonucleotides: dATP, dCTP, dGTP and dTTP each at 0.2 mM); 2.5 mM MgCl2 (Promega, Southampton, UK); 10X reaction buffer [200 mM Tris-HCl (pH 8.8 at 25°C), 100 mM (NH4)2SO4, 100 mM KCl, 1% (v/v) Triton X-100, 1 mg/mL BSA]; 2.5U PfuUltra™ HF DNA polymerase (Stratagene, La Jolla, CA, USA); 10 pmol of 5' phosphorylated sense and antisense oligonucleotide (Sigma-Aldrich, Dorset, UK). The template was denatured for 30 seconds at 95°C. 35 denaturation-annealing-extension cycles were conducted. After completion of the cycling, a final incubation was conducted for 5 min at 72 °C for final annealing and extension.

2.9.4 Restriction Digests of DNA

Restriction enzymes (Promega, Southampton, UK) were used according to the manufacturer's instructions. Briefly, 10-20 U of restriction enzyme was added to 1 µg of plasmid DNA with the appropriate buffer. Mixtures were incubated at 37 °C for 60 minutes and DNA digestion was confirmed by agarose gel electrophoresis.

2.9.5 DNA agarose gel electrophoresis

DNA was analysed using agarose gel electrophoresis. Electrophoresis grade agarose (0.8-2% w/v) (Sigma-Aldrich, Dorset, UK) was dissolved in TAE buffer (0.004 M Tris-acetate, pH 8.0; 1 mM EDTA) with heating. The gel was cooled to 55°C and 10 µl of GelRed (Biotium, Hayward, CA, USA) was added. The mixture was poured carefully into a gel cast containing well comb, to avoid formation of air bubbles. DNA samples were prepared by addition of loading buffer comprised of: 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol, 5% glycerol. Samples were loaded into gel wells, at the cathode end. Gels were run at 120 V for 1 hour until DNA had migrated towards the positive terminal (anode). Bromophenol blue and xylene cyanol migrate at about the same rate as a 500 and 4000 base pair DNA fragment respectively and were used as tracking dyes. DNA molecular weight markers (1 kb DNA ladder, 100 bp DNA ladder; New England Biolabs, Ispwich, MA, USA) were run alongside the DNA samples to allow for determination of approximate DNA fragment size. Gels were imaged under UV light and images were taken by using a gel documentation system (Syngene).

2.9.6 Determination of DNA/RNA concentration

DNA/RNA concentrations were determined using the Nanodrop 2000 (Labtech International Ltd, Heathfield, UK). DNA samples with OD 260/OD 280 ratio between 1.8-2.0 were used in experimental work.

2.9.7 Sequencing of DNA

DNA sequencing analysis was conducted by Eurofins MWG Operon (Ebersberg, Germany). The DNA sample (1.8 µg) was diluted in water with 13 ρmol of primer (annealing at least 50 bp upstream the fragment) made up to a final volume of 50 µl. Samples were shipped in secure lock Eppendorf tubes and analysed using a DNA sequencer. High quality sequencing data from around 500-700 bp fragments was obtained.

2.9.8 Transfection of TR146 and TR146/MUC1 cells

To allow for expression of TAS2R38/Gα16Gust44, TR146 and TR146/MUC1 cells were transfected using plasmid DNA prepared as described above. 0.3µl of LipoJet transfection reagent (Promega, Southampton, UK) was added to the buffer supplied (5µl per well) and the mixture was vortexed for 5 seconds to mix. Endotoxin-free TAS2R38 and Gα16Gust44 DNA (100ng:50ng per well) was added to the mixture, vortexed for 5

seconds and incubated for 10 minutes at room temperature. $1x10^6$/ml cells were resuspended in complete DMEM/F12 Ham media and 50µl/well seeded in a 96-well plate before the transfection reaction mixture was added immediately. Cells were incubated for 24 hours at 37 °C, CO2 (5%) before use in calcium assays. A mock transfection, using an empty vector, was conducted alongside transfection using DNA of interest as a negative control. Empty pcDNA 3.1+ vector (Invitrogen, Carlsbad, CA, USA) was purified as described above and transfected into cells using identical conditions to the TAS2R38/Gα16Gust44.

2.9.9 RNA Extraction

RNA was extracted from cells using the GeneElute Mammalian Total RNA Kit (Sigma, Dorset, UK) following the manufacturer's instructions. Cells were seeded 24 hours prior to RNA extraction at $2.5x10^5$ /ml in 6 well tissue culture plates. For transfected cells, RNA extraction was performed 24-48 hours after transfection. 2.5µl of beta-mercaptoethanol was added to 250µl lysis solution, containing guanidine thiocyanate. Media was removed from cells and wells were washed with phosphate buffered saline ((PBS) 137mM NaCl, 10mM phosphate, 2.7mM KCl, pH 7.4). 250µl of lysis solution was added to each well and the plate was incubated at room temperature for 2 minutes. The lysate was pipetted into a GenElute filtration column. Columns were centrifuged at 12,000 G for 2 minutes. 250µl of 70% ethanol was added to the filtrate and pipetted to mix. Lysates were applied to a GenElute silica binding column and centrifuged at 12,000G for 15 seconds. DNase digestion was performed to eliminate gDNA from the RNA preparations. 10µl RNase free DNase I was mixed with 70µl DNase buffer and applied to the column before incubation for 15 minutes at room temperature (Sigma, Dorset, UK). After two ethanol washes, RNA was eluted with 50µl tris solution (10mM, pH 8.5). The concentration was determined using the NanoDrop and RNA was used for RT-PCR and microarray experiments.

2.9.10 Reverse Transcription

cDNA used in PCR and qPCR was synthesised from RNA preparations using reverse transcription. In a PCR reaction tube, a RT reaction mixture (10µl total) was created, comprised of: 1 µL of oligoDTs (50 µM, Sigma, Dorset, UK), 1 µL of DNTPs (aqueous solution of dATP, dCTP, dGTP and dTTP, each at a final concentration of 10mM, Thermo Scientific, Paisley, UK), 1µg RNA and molecular biology grade water (Thermo Scientific, Paisley, UK). The mixture was incubated for 5 mins at 65°C before rapid

chilling on ice for 1 minute. 1µl superscript iv reverse transcriptase (Thermo Scientific, Paisley, UK) was added, with 4µl buffer (supplied with the superscript kit), 1µl DTT and 4µl molecular biology grade water. The mixture was then incubated at 25°c for 5 minutes then at 50°c for 60 minutes and finally the reaction was inactivated by incubating at 70°c for 15 minutes. Concentration of cDNA samples was determined using the NanoDrop and samples were stored at -80°C between experiments.

2.9.11 qPCR

For quantitative analysis of gene expression in transfected and un-transfected cell lines, qPCR of cDNA samples was conducted. 2 µl of each cDNA sample was added to an Eppendorf and made up to 60µl with molecular biology grade water. In 5 separate Eppendorf's, 30 µl of water was added. The cDNA mix was serially diluted by taking 30 µl from Eppendorf 1 and transferring to Eppendorf 2. This was repeated until Eppendorf 5 and then the final Eppendorf was left as water only as the final standard.

A master mix was created using EvaGreen qPCR mix (containing: HOT FIREPol® DNA Polymerase, ultrapure dNTPs, MgCl2, EvaGreen® dye and ROX dye) (Solis BioDyne, Tartu, Estonia), forward and reverse primer (10µM, housekeeping gene: YWHAZ) and water. EvaGreen dye is a safe, non-mutagenic alternative to SYBER green since it binds DNA but is unable to cross cell membranes and therefore cannot contact genomic DNA in living cells. It works by assuming a looped conformation of two inactive, monomeric DNA-binding dye molecules which shift to a random conformation in the presence of DNA, emitting fluorescence. Samples and standards were prepared in duplicate. Water (nuclease free) was used as a negative control. 1µl of cDNA sample (1µg/µl) or standard was added to 9µl of master mix. Samples were run on the Corbett Rotorgene qPCR machine. The cycling conditions used were a polymerase activation step at 95˚C for 12 minutes followed by 40 cycles of denaturation at 95˚C for 10 seconds, annealing at 60˚C for 10 seconds and extension at 72˚C for 10 seconds. The known DNA concentration of the standards was measured (using the nanodrop) and entered into the Corbett Rotorgene software. For gene(s) of interest the same protocol was followed using primers described in Table 2-2.

Once the run completed a melt analysis was performed to ensure a single peak at 82-84 degrees. Presence of multiple peaks indicated primer-dimerisation and non-specific binding. A Standard curve was generated and R2 values were calculated. The ideal R2

value indicating 100% efficiency is -3.32, however values of -3.1-3.6 (80-110%) are generally acceptable. Relative concentrations analysis was conducted using the Rotorgene software and either the Delta CT Method (relative gene expression levels) or standard curve were used (semi-quantitative concentrations based on known concentration of DNA in standards and relative housekeeping gene expression).

2.9.12 Microarray

TR146 cells were seeded at 2.5 x10^5/ml and grown in T75ml flasks for 24 hours at 37°C, 5% CO2. Two identical flasks were prepared, and one flask was treated with 100µM PTC, dissolved in DMEM-F12 media, every 2 hours for 8 hours. The other "control" flask was treated in the same way however, PTC was omitted. RNA was extracted from both flasks using the GeneElute Mammalian Total RNA Kit (Sigma, Dorset, UK), as described above, and sent to the King's College London Genomic Centre (Waterloo Campus, London, UK) for microarray analysis. A full list of expressed genes was provided (log_2 scale) as well as fold change in gene expression following PTC treatment.

2.10 Calcium Response Assays

2.10.1 Confocal Microscopy

TR146 cells were grown in coverslip bottom dishes (MatTek, Ashland, Massachusetts, USA) pre-incubated with 50 µL/well of a fluo4-AM solution consisting of: 5 µL fluo-4AM (Life Technologies, Paisley, UK) (prepared at a concentration of 2.5 mM in 50% Pluronic F-127 (Life Technologies):50% DMSO) in 5 mL saline solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4). Plates were incubated for 60 minutes at 37°C, 5% CO2. During incubation, fluo4-AM enters the cell where cellular esterases catalyse the hydrolysis of the AM-ester, releasing fluo4 which fluoresces in the presence of free Ca^{2+} ions. Excess dye solution was removed, and fresh saline solution added before baseline fluorescence was viewed on an A1R confocal microscope (Nikon, UK). Cells were imaged at 40X with an excitation of 480nm and an emission of 525nm for 1 minute before addition of agonist (PTC and PROP, prepared in saline solution) 1:1 diluted into saline already present on cells. Imaging was continued for a further 2 minutes before addition of ionomycin (10µM) and further imaging for 1 minute to establish a maximal fluorescent response. Ionomycin response was set at 100% for data analysis and fluorescence at

baseline or in response to agonists was shown as a percentage of the ionomycin (maximum) response to allow normalisation between dishes, accounting for uneven cell distribution and calcium dye loading. The fluorescence intensity was quantified using NIS elements software (Nikon, UK). The fluorescence in approximately 10 individual cells was analysed and averaged to provide response profiles from each dish.

2.10.2 FLEX Station Intracellular Calcium Measurements

Intracellular calcium measurements were conducted using fura-2/fluo-4 staining of cells. For the assays, TR146 MUC1 cells were seeded on black, clear bottom 96-well culture plates (Greiner Bio-One Ltd, Gloucester, UK), at a density of 5×10^5 cells/ml. SCC090 were seeded at a density of $1x10^6$/ml, incubated for 24 hours until >80% confluency was achieved.

TR146/MUC1 and SCC090 cells were pre-incubated with 50 µL/well of a fura-2-AM solution consisting of: either 5 µL fura-2-AM or 5µl fluo-4AM (Life Technologies, Paisley, UK) (prepared at a concentration of 2.5 mM in 50% Pluronic F-127 (Life Technologies):50% DMSO) in 5 mL saline solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 2 mM CaCl2, 10 mM glucose and 10 mM HEPES, adjusted to pH 7.4). Plates were incubated for 60 minutes at 37°C, 5% CO2. During incubation, the fura-2-AM/fluo4-AM enters the cell where cellular esterases catalyse the hydrolysis of the AM-ester, releasing fura-2/fluo4 which fluoresces in the presence of free Ca^{2+} ions. Excess dye solution was removed, and fresh saline solution added and baseline fluorescence readings (fura-2 AM = excitation 340 nm/emission 525 nm, fluo-4 AM = excitation 480nm/emission 525nm) were taken for 1 second using a FlexStation 3 (Molecular Devices, San Jose, CA, USA). The automatic pipetting system on the FLEX station was set to add the taste compound after 30 seconds of plate reading and readings were taken every 5 seconds for 1minute following compound addition. Ionomycin (10uM) was added by the FLEX station, as a control to elicit maximum response in cells and allow for normalisation between wells, after 1 minute and readings continued for 30 seconds more. Data were analysed using Softmax Pro software and expressed as the ratio between excitation and emission spectra (fura-2) or the difference between maximum fluorescence and baseline fluorescence (expressed as a ratio of ionomycin fluorescence) (fluo-4).

For measurement of responses to caffeine, probenecid (Sigma, Dorset, UK) (1mM final dilution, 2µl of 500mM stock, prepared in 1N NaOH added per 1ml saline) was added to the fluorescent dye/saline mixture and used to prevent leakage of calcium indicator dye. However, due to the reported allosteric inhibitory action of probenecid on TAS2R38 receptors (Greene et al. 2011), it was not used in PTC experiments except as an inhibitor to control for TAS2R38 independent responses. Some dye leakage did occur in cells where probenecid was not added and as a result, the fluorescence readings were lower in those experiments.

2.11 Statistics

Microsoft Excel (Version 1804, Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA) was used for data analysis and generation of graphs. SPSS version 24 was also used for statistical analysis (IBM Analytics, Armonk, NY, USA). The data was tested for normal distribution using the D'Agostino & Pearson's normality test. Data which was not normally distributed was analysed using non-parametric tests. Data which was normally distributed was analysed with parametric tests. The student's t test was selected in the case of testing difference between 2 variable, even in the case of abnormal distribution when there was a great enough 'n' number to allow use of the parametric test (Lumley et al. 2002). Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****. Bi-variate linear regression analysis was conducted for correlations analysis. The Pearson's linear regression was used for normally distributed data and Kendall's tau b technique was used for data which was not normally distributed. A correlation coefficient >0 was regarded as a positive correlation and a coefficient <0 as a negative correlation with a P value <0.05 indicating significance.

3 Characterisation of Salivary Responses to Taste, TRP Stimulation and Olfaction in Younger and Older Adults

N.B. The content of this Chapter of the thesis is modified from a publication entitled "Altered salivary flow, protein composition and rheology following taste and TRP stimulation in young and older adults" by Pushpass, R. et al. in which I am the leading author. The publication was submitted 27/04/2018 to Frontiers in Physiology Journal, edited by Professor Anis Larbi, and is currently under peer review.

3.1 Introduction

The senses of smell and taste are both governed by receptor cells in the olfactory bulb or taste bud respectively. Signals from these cells upon stimulation from odorant/tastant molecules are sent to the thalamus in the brain where they combine to give the sense of flavour (Frank et al. 1993).

There are 5 basic tastes comprising sweet, umami, bitter, sour and salt (Roper 2013). Type ii taste bud cells have G-protein-coupled receptors (GPCR's) on their surface which bind taste molecules (Chandrashekar et al. 2006). Sweet tastants bind to T1R2 and T1R3 receptors, umami to T1R1 and T1R3 and bitter to heterologous T2R/TRB complexes (Zhang et al. 2003, Zhao et al. 2003a). Interaction of taste molecules with the receptor activates G alpha gustducin, a Gαi protein, triggering a cascade of reactions culminating with activation of transient receptor potential channel 5 (TRPM5) and release of ATP which activates adjoining nerves (Huang et al. 2011, Huang and Roper 2010). In contrast, sour and salt tastants follow different transduction pathways with diffusion of protons or salt ions, respectively, through ion channels in the cell membrane of type iii receptor cells (Chang, Waters and Liman 2010, Ishimaru and Matsunami 2009, Kataoka et al. 2008, Lyall et al. 2001, Chandrashekar et al. 2010).

There is a link between age and the decline of taste acuity, often coupled with decreased olfactory sensation, particularly retro nasal (via the mouth) (Stevens and Cain 1986). Bitter taste may be most affected by age (Yoshinaka et al. 2016), but age related increases in taste thresholds have also been associated with salt, sour and umami, with rate of taste loss being dependent on which substance is used in testing (Mojet et al. 2001, Schiffman et al. 1991, Schiffman et al. 1994b, Jiang et al. 2016). Sweet taste is reported to be largely unaffected with age (Mojet et al. 2001, Yoshinaka et al. 2016).

Taste and smell sensation appear to decline noticeably at similar rates from the age of 60 (Ng et al. 2004, Schiffman 1993) and in the case of central processing of taste in the brain, perhaps even as early as middle age (45 to 54 years old) (Green et al. 2013). This can have serious implications for the health and quality of life of an elderly individual. Their nutritional status may be impaired as a result of reduced food intake because of reduced enjoyment when eating (Schiffman 1997). Taste loss may also result in unhealthy eating behaviours such as increased salt intake to heighten flavour in meals (Stevens et al. 1991) or eating more sugary foods (Gilmore and Murphy 1989). Loss of sense of smell can lead to difficulty in detecting out of date foods, or failure to notice smoke or a gas leak (Boyce and Shone 2006).

In this study a bitter tastant was used to represent a basic taste with noticeable differences in sensation between age groups. Umami taste thresholds may be less affected by ageing than salt taste (Dermiki et al. 2013). As such, umami taste is of interest in intervention studies aimed at improving palatability of food and increasing appetite for older adults, as it does not have the negative health impact of adding salt and sugar (Schiffman et al. 1991, Masic and Yeomans 2014, Schiffman 1998). Umami tastants may also increase salivary flow and, as reduced taste function is correlated with both hypo-salivation and poor oral health, could be useful in improving taste function in older adults (Sasano et al. 2010, Schiffman 1998). Supplementing meals with umami flavours has been shown to enhance consumption in older adults even when liking of the meal was unchanged (Dermiki et al. 2015). Addition of both MSG and natural umami flavours, to foods, have been shown to have positive effects on energy consumption in older adults (Dermiki et al. 2014, Dermiki et al. 2015). Post-ingestive effects of nutrient providing tastants, including umami compounds which are associated with protein and amino-acid intake, can encourage consumption (Prescott 2004, Masic and Yeomans 2013). Therefore, the ability to perceive MSG in foods is not necessarily a requirement for its positive effects on food consumption. Indeed, liking of umami enhanced foods may be greater in those with reduced taste sensitivity compared to lower taste threshold individuals (Dermiki et al. 2013). This may be due to individuals with high taste sensitivity finding the taste of enhanced meals overpowering, while for those with sensory loss, higher levels of tastants may be more pleasant. MSG was therefore also used in this study to investigate suprathreshold sensitivity to a basic taste modality, which may be useful in encouraging nutrient intake, in older adults, who are at risk of malnutrition.

PAV/AVI polymorphism of TAS2R38 gene influences the ability to detect bitter taste compounds which contain a thiourea group, including Propylthiouracil (PROP) and Phenylthiocarbamide (PTC) (Kim and Drayna 2005). The presence of Pro, Ala and Val at positions 49, 262 and 296 of the protein, respectively, is associated with taste while Ala, Val and Ile at the same positions associate with non-taste. Relative numbers of PTC/PROP tasters/non-tasters are not significantly different among different age groups (Abraimov and Mirrakhimov, 1979; Whissell-Buechy, 1990). However, PTC/PROP taster status has been correlated to taste sensation in general since tasters have overall greater sensitivity to bitter and sweet tastants (Chang et al. 2006, Bartoshuk, Duffy and Miller 1994, Hong et al. 2005a). Also, PCT taste threshold could be negatively correlated with thresholds for all of the basic tastes (Hong et al. 2005a). Furthermore, capsaicin sensitivity is also greater in PTC/PROP tasters (Bartoshuk et al. 1994, Nolden, McGeary and Hayes 2016). This may be due to a greater density of taste buds on the tip of the tongue (Bartoshuk et al. 1994). To examine this theory in the context of ageing, PTC taster status of a sub-set of the older and younger participants was tested and correlated to overall taste function.

Aside from the basic tastes, other compounds in foods can elicit responses in the taste buds. Heat, pungency, cooling, astringency and fattiness are all sensations felt on the tongue but are not considered "tastes". Compounds such as capsaicin (chilli) cause a stinging/burning sensation in the mouth due to direct activation of TRPV1 channels, expressed by epithelial cells on the tongue and oral mucosa (Marincsák et al. 2009, Wang et al. 2011). TRPM8 channels are expressed by trigeminal ganglia nerves in the tongue and in oral epithelial cells (Abe et al. 2005, Wang et al. 2011) and are activated by cold temperatures as well as by menthol and cooling agents (Peier et al. 2002, Reid et al. 2002).

Reduction in sensitivity to TRP agonists could also influence the effect that ageing has on hedonic aspects of eating for elderly people (Fukunaga et al. 2005). Older adults display increased sensitivity thresholds and reduced effect of repeated exposure to menthol compared to younger adults for olfaction, oral trigeminal sensation and in foods (Forde and Delahunty 2002, Koskinen et al. 2003, Murphy 1983, Kremer et al. 2007b). However, capsaicin sensitivity does not appear to be lost with ageing, even in those who have loss of basic tastes (Fukunaga et al. 2005, Forde and Delahunty 2002).

Therefore, certain TRP receptor stimulants may evoke oral sensations which could increase the enjoyment of eating for older individuals who have reduced taste ability.

Self-reported taste and smell dysfunction does not always correlate with psycho-physical testing (Soter et al. 2008). In older adults, cognitive function can affect the ability to self-report sensory disorders (Steinbach et al. 2010, Nordin, Monsch and Murphy 1995). Older adults frequently under-report smell loss (Nordin et al. 1995). Additionally, individuals with olfactory disfunction may assume they also have taste loss since the ability to detect flavour largely depends upon olfaction (Soter et al. 2008). Therefore, questionnaires used to assess taste and smell loss should make use of specific, focussed questions for example "can you detect the taste of sugar in chocolate?" (Soter et al. 2008). Additionally, questions regarding food behaviour can shed light on changes in dietary habits which may have been caused by taste or smell loss for example adding more salt to food or increasing intake of sugary foods. However, the effect of "social desirability" bias cannot be excluded since people are less likely to admit to eating behaviours which are negatively perceived in society, such as sugar consumption (Van de Mortel 2008, Mertz et al. 1991). Social desirability bias has shown to be greater in the older population, with a tendency to report themselves more favourably, perhaps because of negative stereotypes about old age in western society (Dijkstra, Smit and Comijs 2001). Therefore, the present study used questions designed to control for social desirability bias. This included asking questions which were un-related to taste, smell and food behaviours so that participants would not be influenced by the knowledge that the focus of the questionnaire was sensory loss and eating habits. This study made use of a questionnaire to gain insight into the changes in eating habits occurring in ageing and to see if self-reported taste and smell loss could be correlated to perception data from the physical testing.

Saliva is a hypotonic fluid originating in the oral cavity with low concentrations of proteins, electrolytes and nitrogenous products. It aids transduction of tastants by solubilising and facilitating their movement to the taste pore where they may bind to receptor cells (Almeida, P. 2008) (Bradley et al. 2003). Removal of the major salivary glands results in pathological changes in the taste tissue and significant decrease in taste sensitivity (Matsuo et al. 1997, Nanda and Catalanotto 1981). There are three main salivary glands in the oral cavity. The parotid, which secretes a watery fluid and the

submandibular and sublingual glands which secrete viscous mucin rich saliva (Proctor 2016).

Surface-bound MUC1, on oral epithelial cells, may facilitate muco-adhesion of the salivary pellicle by binding to salivary MUC5B (Ployon et al. 2016, Gibbins et al. 2015). This layer of saliva protects the oral surfaces, including the tongue, bathing the taste buds and providing a trophic stimulus. The rheological properties of saliva are important for mouth feel, muco-adhesion and protective function of saliva. Reduced viscoelasticity of saliva can be correlated with reported dry mouth in xerostomic patients (Chaudhury et al. 2016). Viscoelasticity of stimulated whole mouth saliva is dependent on stimuli (Stokes and Davies 2007, Vijay et al. 2015). Previous studies investigating viscoelasticity of stimulated saliva have largely focussed on different methods of stimuli using a single taste, smell or mechanical stimulus, but this study used multiple taste and TRP compounds to see how different compounds might affect salivary viscoelasticity.

Saliva has antibacterial and lubricating properties which protect the oral mucosa including the tongue and provides growth factors for renewal of taste buds, (Mese and Matsuo 2007, Morris-Wiman et al. 2000). In particular, proteins from the cystatin family are involved in anti-bacterial protection in the mouth via inhibition of cysteine proteinases, involved in periodontal disease (Baron et al. 1999, Ito et al. 2008, Ganeshnarayan et al. 2012). Increased cystatin S in saliva has previously been associated with reduced sensitivity or greater acceptance for bitter taste (Dsamou et al. 2012, Morzel et al. 2014). Protease inhibitory action of cystatin S was the proposed mechanism for this since reduced protease activity may result in a thicker saliva which acts as a barrier to tastants. Conversely, increased protease activity may result in a thinner saliva and lead to hypersensitivity to taste compounds (Dsamou et al. 2012).

Carbonic anhydrase VI (CAVI) is a metalloprotein thought to act as a taste cell growth factor and lower levels in saliva have been linked with taste loss of multiple aetiologies (Shatzman and Henkin 1981, Igarashi et al. 2008). Salivary CAVI has also been positively correlated with increased sensitivity to bitter and sweet taste compounds, especially for PROP/PTC (Rodrigues et al. 2017, Walliczek-Dworschak et al. 2017). Although there is some disagreement in the literature regarding the role of CAVI in PTC/PROP sensitivity, with Feeney et al finding no association (Feeney and Hayes 2014). Salivary CAVI may function as a growth factor, important for the development

and maintenance of taste buds (Thatcher et al. 1998, Padiglia et al. 2010, Calò et al. 2011, Melis et al. 2013b). As such, cystatin S, as an anti-bacterial protein and CAVI, as a potential taste bud growth factor, were quantified in UWMS from older and younger adults. This gave information about the effect of salivary protection against microbial attack and taste bud growth factors specifically on age related taste loss.

Additionally, salivary pH may correlate to taste sensation. Increased salivary pH has shown to lead to increased sweet taste sensitivity in both animal models and in humans (Matsuo and Yamamoto 1990, Aoyama et al. 2017). Therefore, this study also measured salivary pH and correlated this with taste, TRP and odour responsiveness to see whether pH of saliva could be a predictive measure for sensory loss. Furthermore, salivary viscoelasticity may be related to pH. Mucins form gel phases at lower pH because acidic pH causes protonation of the carboxylates of the salt bridges, leading to their breakage and exposing hydrophobic regions which associate non-covalently with adjacent molecules (Bansil and Turner 2006, Wagner et al. 2017). Higher salivary pH may also be related to bicarbonate concentration. Bicarbonate is a calcium chelator which removes calcium from cross-linked mucin gels and so, facilitates gel dispersal (Chen et al. 2010). For this reason, higher salivary pH may be related to reduced viscoelasticity.

Dehydration, medication and diseases common in older people can reduce the salivary flow, especially un-stimulated whole mouth saliva (UWMS) which may be a factor in age related taste loss (Nagler and Hershkovich 2005a, Affoo et al. 2015). Parotid salivary flow is mostly retained while submandibular/sublingual flows (responsible for 70% of resting salivary flow and 50% of stimulated) are significantly reduced (Affoo et al. 2015). Protective factors such as salivary mucins, immunoglobulins and enzymes are known to be reduced in saliva of older adults (Denny et al. 1991, Navazesh et al. 1992, Vissink et al. 1997). This may prevent lubrication of oral surfaces including the taste buds and hinder dissolution and transport of tastants to receptor cells. Few studies have investigated the effect of ageing on rheology of saliva although it has been suggested that advanced age leads to increased viscoelasticity (Zussman et al. 2007).

Taste thresholds are often based on individual perception and are not a definitive value for taste responses especially in ageing populations where cognitive decline is a factor (Engelen et al., 2003). Despite this, determining the population group of those who perceive themselves to have taste/smell loss is still of value since individual perceptions

of sensory loss are an important factor when developing therapies (Bhattacharyya and Kepnes 2015). Moreover, sensory testing in older adults should utilise simple methods to avoid confusion and fatigue as well as considering visual and hearing impairments common in older adults (Methven, Jiménez-Pranteda and Lawlor 2016). A labelled scale may be more suitable for assessing sensory perceptions of older adults, as visual analogue scales may be confusing for those with cognitive impairment (Methven et al. 2016). As such, this study used a simple design for sensory testing involving assessment of intensity perceptions of one supra-threshold concentration of each stimulant and using a simple labelled scale which has semantic descriptors, to minimise subject confusion.

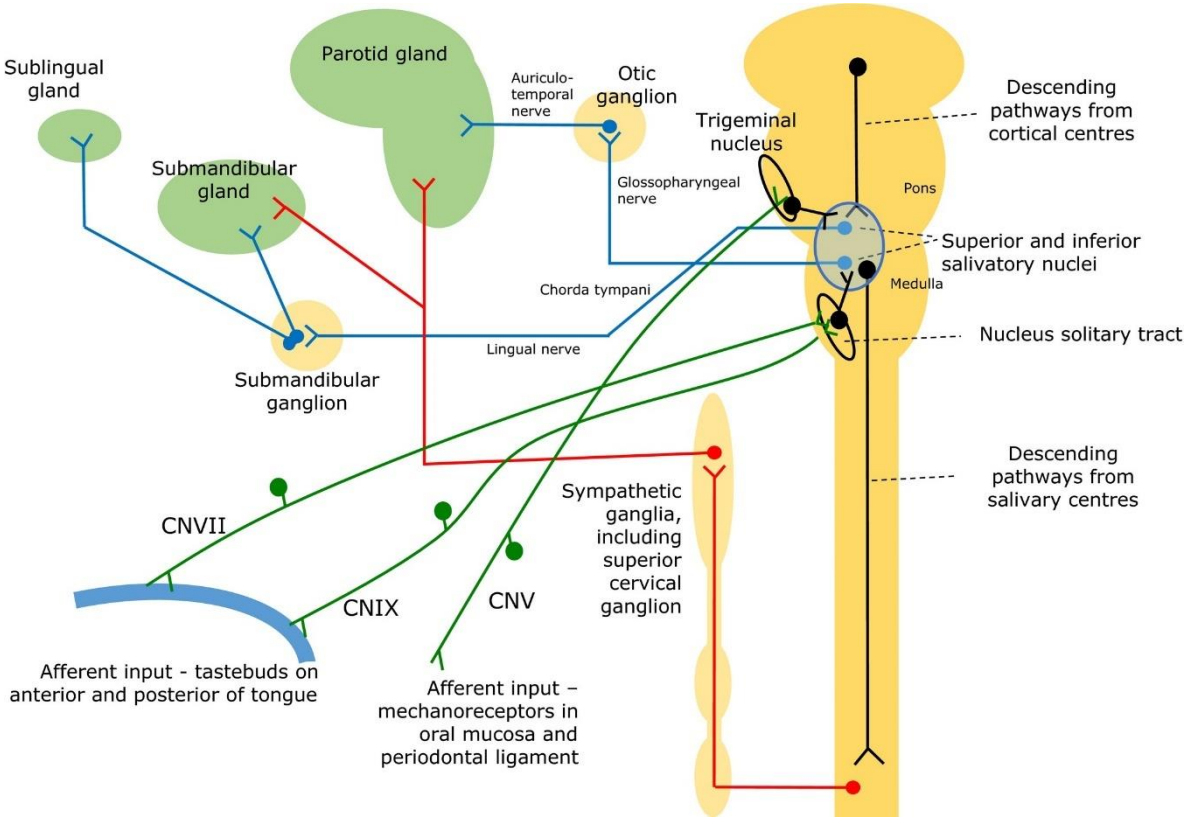


Figure 3-1 (Proctor 2016) Salivary Reflex Response is Governed by Nerves

Saliva is secreted as a response to several stimuli including taste, smell and mechanical stimulation such as chewing (Hector 1999). Previous studies have shown that saliva is secreted as a reflex response to all the basic tastes, with the greatest secretions elicited by umami and sour tastants (Hodson and Linden 2006). Parotid gland salivary secretion has been shown to provide an accurate measure of gustatory responses (Chauncey and Shannon 1960) but collection of whole mouth saliva is less invasive and more efficient than collecting saliva from individual glands. Additionally, taste compounds likely

stimulate different glands in different proportions (Stokes and Davies 2007, Vijay et al. 2015), therefore collection from only the parotid gland may lead to false results if a tastant preferentially stimulates the submandibular/sublingual glands. A reflex salivary response has also been shown for the labial minor salivary glands, in response to all five basic tastes, and could be positively correlated with taste perceptions in healthy adults (Satoh-Kuriwada et al. 2018). Salivary reflex response to taste likely recruits parasympathetic nervous system pathways, because increased labial blood flow was also correlated to taste perceptions and labial salivary flow (Satoh-Kuriwada et al. 2018). TRP agonists also induce salivary secretion, above basal levels (Nasrawi and Pangborn 1990, Lawless 1984).

Taste, smell, TRP agonists and mechanical stimulation stimulate afferent nerve fibres of the facial, glossopharyngeal and trigeminal nerves (Proctor 2016). The solitary tract nucleus sends signals to the salivary centres in the brain (superior and inferior salivary nuclei in the medulla oblongata) following stimulation of the facial and glossopharyngeal nerves (Figure 3-1 (Proctor 2016). The salivary nuclei are influenced by these central nerves from other nuclei in the brain (Ishizuka et al. 2010). Efferent nerve signals are transmitted from the salivary nuclei to the submandibular ganglion via the chorda lingual nerve resulting in secretion from the submandibular and sublingual glands (Figure 3-1). The parotid gland is stimulated via efferent signals in the tympanic branch of the glossopharyngeal nerve to the auriculo- temporal nerve (Figure 3-1). Stimulation of afferent nerves following taste, TRP and smell exposure, leads to a salivary response via nerve signals sent via the salivary nuclei. For this reason, the present study used salivary response as an objective measure of taste as well as subjective taste perceptions to provide a comprehensive overview of age related loss in oral sensations without the influence of individual bias.

The aim of this part of the study was to investigate the effect of age on subjective (perception and self-reported questionnaire) and objective (stimulated salivary flow) responses to TRP stimulants, odours and basic tastants. UWMS and SWMS were characterised in older and younger participants for key properties which may be relevant to oral sensory sensation such as extensional rheology, pH and protein composition. This enabled understanding of the ways ageing might influence salivary characteristics and impact oral sensory sensation. An additional aim was to see whether age related sensory loss could be correlated with self-reported sensory loss and changes in self-

reported eating behaviour. Since most previous taste testing studies have used subjective measures of taste perceptions which are prone to subject bias and are influenced by individual experiences, this study aimed to assess taste responses in an objective manner by using salivary responses to oral stimuli. This also allowed for collection of biological samples which could be further analysed for markers of sensory loss. Furthermore, many previous studies have investigated age related taste loss using increasing concentrations of tastants to determine detection or recognition thresholds. This may not be relevant in real life since the concentrations of tastants present in foods is often much greater than threshold concentrations. As such, this study used one supra-threshold concentration of each stimuli to determine sensitivity to concentrations of oral stimuli which are representative of those experienced in real life food consumption. This also reduced the time burden for participants and simplified the study protocol.

3.2 Methods and Materials

3.2.1 Study Group

The study group was as described in chapter 2, Methods and Materials.

3.2.2 Rheological Analysis

Extensional Rheology (ER) was analysed in saliva samples as described in chapter 2.

3.2.3 Compositional Analysis

pH of saliva was measured only in a sub-set of the study group (60+, n=10, 18-30, n=14) due to difficulty obtaining a reliable, portable method for assessing pH at the time of saliva collection.

Total Protein Concentration

The Bicinchoninic acid assay (BCA assay) was used to determine the total protein concentration of samples, described in chapter 2.

Gel Electrophoresis

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to analyse protein composition of samples (described in chapter 2). A control sample of un-stimulated whole mouth saliva (UWMS) from one healthy donor (aged 27 years) was included in each gel to allow for normalizing between gels.

Western Blotting

To detect antibodies for carbonic anhydrase VI and cystatin S gels were transferred, using western blotting, onto nitrocellulose membranes via electrophoresis. Details of the technique and anti-bodies used can be found in chapter 2.

3.2.4 Normalising Data for Analysis

When comparing between groups, taste and TRP SWMS flow rate, Spinnbarkeit, total protein and mucin levels were normalized to the corresponding result for water rinse (water and 1% PG) control in each individual, to control for the effect of rinsing and use of carrier (PG) to dissolve some of the compounds. Olfactory SWMS results were normalized to the corresponding UWMS result. When comparing within groups results were not normalized. Rinsing with the water rinse caused increased salivary flow

compared to UWMS which was significantly greater in the younger group (see Appendix 4 for raw data) and therefore normalising all data to water rinse response showed age related effects of the stimulating compound only. The WMS ER and protein content were not significantly higher in any of the SWMS compared to UWMS (data not shown). Mouth rinsing disturbs the salivary pellicle, and as such the mucins contained within would probably be expectorated along with the mouth rinse solution. This could therefore reduce the mucin content of the SWMS subsequently collected and therefore UWMS has a greater ER and mucin content. For these reasons, data from each individual's taste/TRP mouth rinses were analysed as a ratio to the water rinse control (WMS), to show the effect is induced by specific stimulants and is not a result of mouth rinsing. Since the menthol odour stimulation did not involve a mouth rinse, it was appropriate to compare the results from this to unstimulated WMS. This additionally normalises the data to each individual's "baseline" response and thus reduces the impact of inter-individual variation.

Results depicted on graphs show data which has been normalised to water rinse and therefore significance between groups donated by a *. Significance from baseline within groups is denoted by a + and shown on the same graphs, however this data was not normalised to water rinse before being analysed for significance.

For SDS-PAGE gel electrophoresis and western blotting, data was normalised to a control saliva sample from one donor (aged 27 years old). The same saliva sample was run on all gels to allow normalisation between gels. Western blotting band intensities were normalised to the corresponding amylase band intensity (also determined by western blotting) on the same blot as a protein loading control.

3.2.5 Statistics

Microsoft Excel (Version 1804, Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA) was used for data analysis and generation of graphs. SPSS version 24 was also used for statistical analysis (IBM Analytics, Armonk, NY, USA). The data was tested for normal distribution using the D'Agostino & Pearson's normality test. Data which was not normally distributed was analysed using non-parametric tests, Friedman test with Dunn's multiple comparisons for significance within groups. Data which was normally distributed was analysed with one-way ANOVA and Holm-Sidak's multiple comparisons test. An

independent student's t test was used for difference between groups. The student's t test was selected despite lack of normal distribution since there was a great enough subject number to allow use of the parametric test (Lumley et al. 2002). Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 **** shown as * (significance between groups) and + (significance from baseline within groups). Bi-variate linear regression analysis was conducted for correlations between salivary parameters and sensation. The Kendall's tau b technique was used to calculate a correlation coefficient and a coefficient of 0 regarded as no correlation, a coefficient of 1 regarded as a positive correlation and a coefficient of -1 as a negative correlation.

3.3 Results

3.3.1 Comparative Subjective Taste and Odour Perceptions in Older and Younger Adults

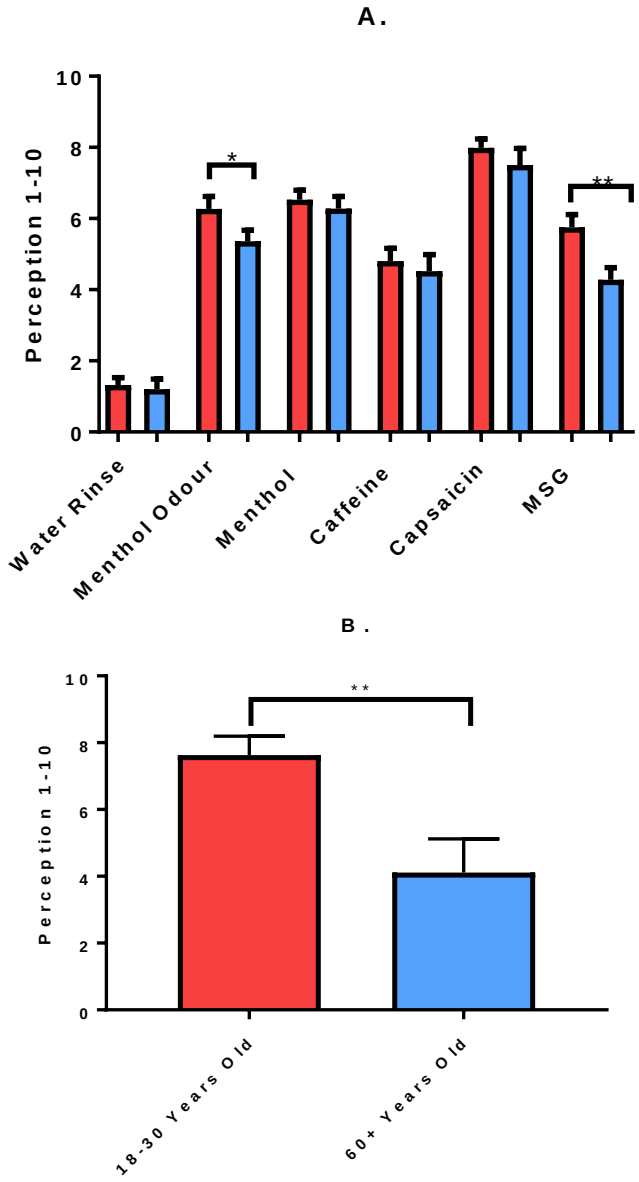


Figure 3-2 Taste, TRP and Odour Perceptions in Older and Younger Adults

A. Average (+/-SEM) perceived intensity of taste and TRP agonists by subjective measurement on a scale of 0-10 in younger (18-30 years) and older (60+ years) subjects. 18-30 years old n=31, 60+ years old n=25. P = < 0.05 * P = < 0.01 **. B. Average (+/-SEM) perceived intensity of PTC filter paper strips by subjective measurement on a scale of 0-10 in a subset of the same younger (18-30 years, n=20) and older (60+ years, n=9) subjects. P = < 0.05 * P = < 0.01 **. Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups).

To assess the effect of participant age on subjective taste, smell and TRP sensation, a labelled rating scale was used from 0-10, no sensation at all to strongest imaginable sensation. The average (+/-SEM) perception of menthol smell and the MSG mouth rinse were significantly greater in the younger participant group compared to the older 6.27 +/-0.35 and 5.36 +/-0.31 ($p<0.05$) and 5.76 +/-0.36 (18-30) and 4.28 +/-0.34 (60+) ($p=<0.01$) respectively (Figure 3-2 A). Mouth rinses of water (control) and other taste/TRP solutions were not perceived differently between the two age groups.

To assess the effect of ageing on perception of PTC, a TAS2R38 agonist, filter paper strips were prepared with 50mM PTC as previously described (Zhao et al. 2003b). The average (+/-SEM) perception of PTC was significantly greater in the younger group compared to the older with a mean perception of 7.6 +/- 0.6 (n=20) and 4.1 +/- 1.0 (n=9) respectively (Figure 3-2 B).

Table 3-1 Kendall's tau b correlation between PTC response and overall taste sensitivity in older and younger adults.

	Age of Participant			PTC response 1-10
Kendall's tau_b	18-30 Years Old	is taste sensitivity low or high	Correlation Coefficient	.515*
			Sig. (2-tailed)	.010
			N	20
	60+ Years Old	is taste sensitivity low or high	Correlation Coefficient	.202
			Sig. (2-tailed)	.515
			N	9

To see whether PTC responsiveness could be linked with taste sensitivity, a linear regression (Kendall's tau b's correlation) was conducted between PTC responses and taste sensitivity. All participants were grouped into high and low taste sensitivity, as previously described (Rodrigues et al. 2017). Briefly, an individual was assigned as low sensitive, and given a value of 1 for a stimulant when they had perception which was lower than the median of all perceptions observed within that age group. An individual was assigned as high sensitive, and given a value of 2, for a stimulant when their

perception was equal to or higher than the median. An average of all stimulants was taken and individuals with an average value >1.5 were assigned high sensitivity status, those with an average value of <1.5 were assigned low sensitivity status. PTC status could be positively correlated to taste sensitivity in younger adults but not in older adults (Kendall's tau b's correlation coefficient 0.51, p=0.01, Kendall's tau b's correlation coefficient 0.20 p=0.52 respectively) (Table 3-1).

3.3.2 USWMS Flow Rate and SWMS Response to Taste, TRP and Olfactory Stimuli

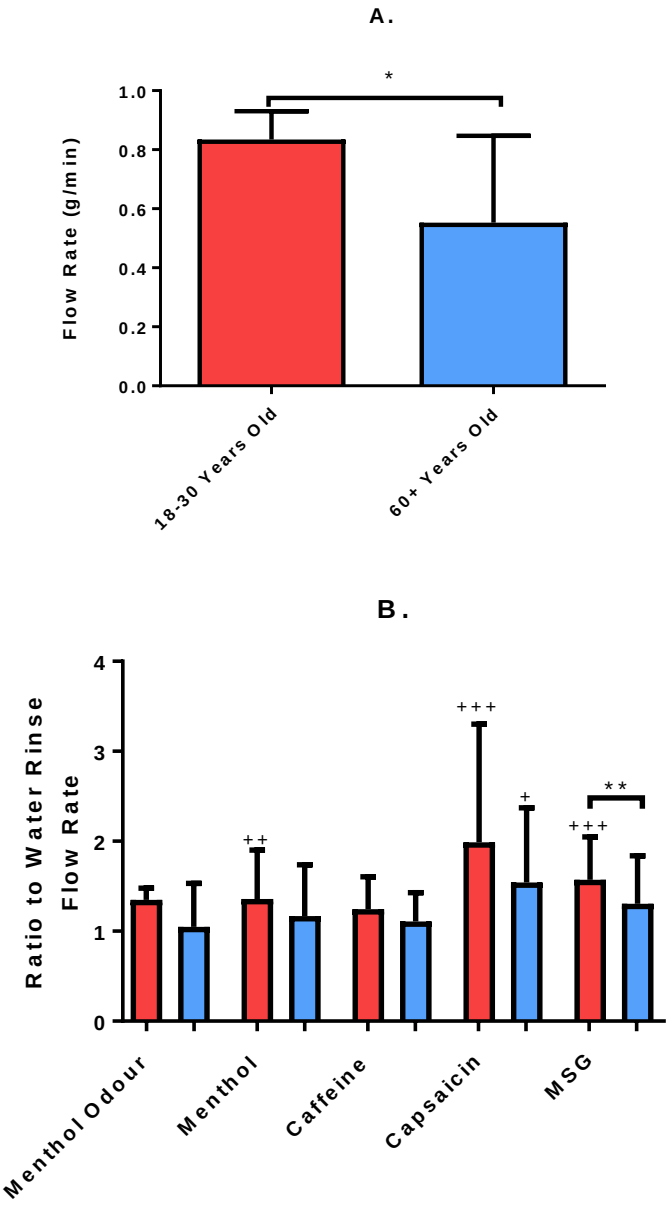


Figure 3-3 UWMS and SWMS flow Rates in older and younger Adults

A. Average (+/-SEM) UWMS flow rate of younger (18-30 years, n=31) and older (60+ years, n=25) subjects. P = < 0.05 */+ P = < 0.01 **/++ P = < 0.001 ***/+++. B. The average (+/-SEM) WMS flow rate following 1 minute of 1ml mouth rinse of taste and TRP agonists or 1-minute smelling of menthol in younger (18-30 years) and older (60+ years) subjects. Flow rate (g/min) following taste/TRP stimulation expressed as a ratio of flow rate (g/min) following water rinse. Flow rate (g/min) following menthol odour stimulation expressed as a ratio of resting flow rate (g/min). 18-30 years old n=31, 60+ years old n=25. Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups).

The older group had a significantly lower UWMS salivary flow rate, 0.55 +/- 0.60g/min compared to 0.83 +/- 0.10g/min in the younger group ($p<0.05$) (Figure 3-3 A). Saliva was secreted as a reflex response to stimulus including taste and smell and so SWMS flow rate was calculated as an objective measure of taste response. The SEM for UWMS flow rate was greater for the older group suggesting more inter-individual variation in salivary flow rates amongst this age group. When results were normalized to the water rinse stimulated flow rates (g/min), average salivary flow rate (+/-SEM) following mouth rinse of MSG was significantly greater in the younger group compared to the older, 1.57 +/- 0.09 and 1.31 +/- 0.11 ($p<0.05$) (Figure 3-3 B)). In both age groups, there was a significant increase in salivary flow rate following mouth rinses with capsaicin from salivary flow rate after rinsing with the water rinse control, up to 0.62 +/-0.05g/min from 0.48 +/-0.05g/min in the older ($p<0.05$) and up to 2.37 +/-0.41g/min from 1.32 +/-0.22g/min in the younger ($p<0.01$). MSG mouth rinsing also induced significantly increased salivary flow in the younger group up to 2.02 +/-0.36g/min (MSG, $p<0.001$). Menthol odour and caffeine did not evoke significantly increased WMS flow rate in either age group.

3.3.3 Salivary pH

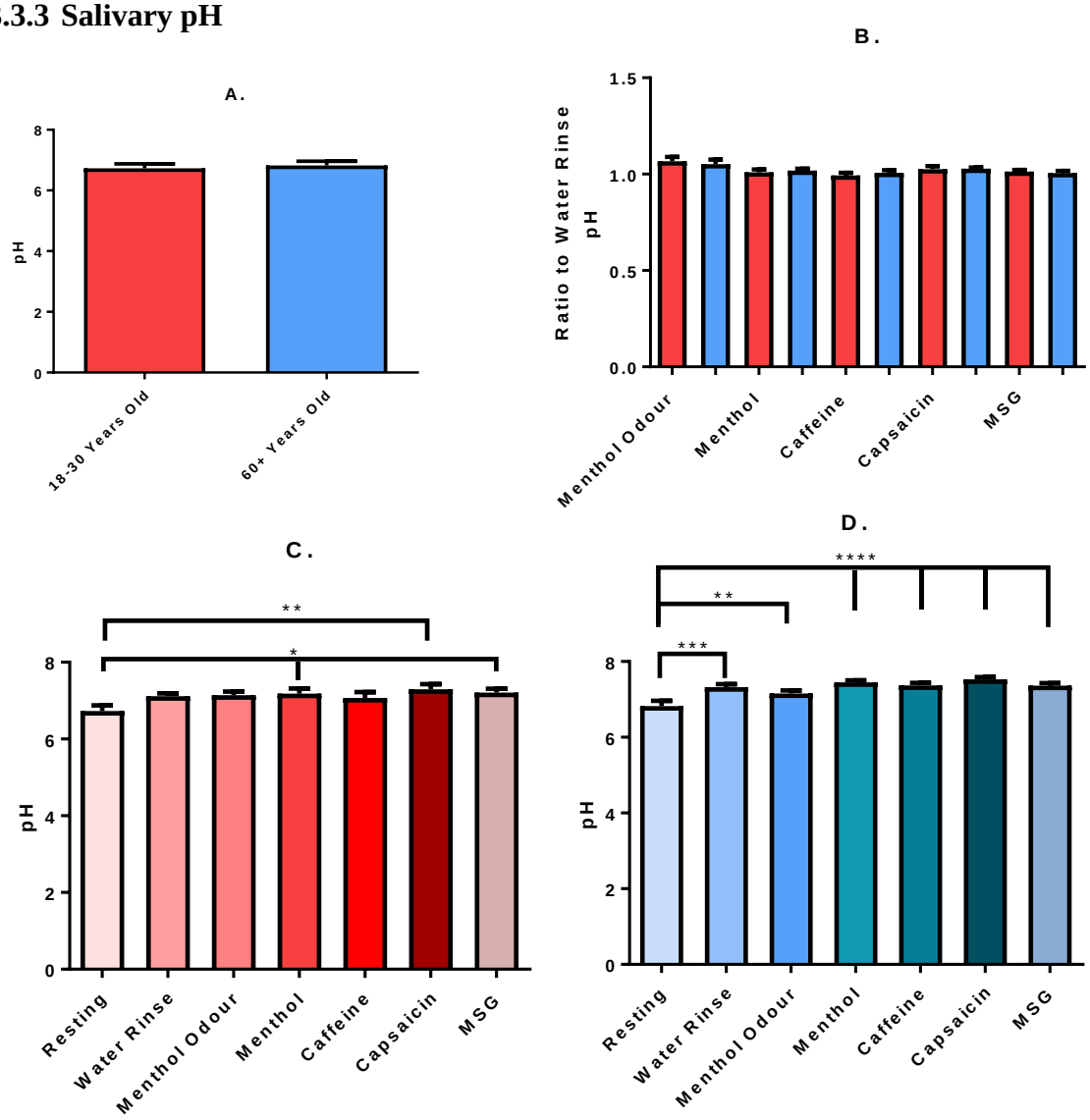


Figure 3-4 pH of UWMS and SWMS in older and younger adults

A. Mean (+/-SEM) pH of UWMS in a subset of the younger (18-30 years old, n=14) and older (60+ years old, n=10) adults. B. Mean (+/- SEM) pH of WMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol. pH following taste/TRP stimulation expressed as a ratio of pH following water rinse. pH following menthol odour stimulation expressed as a ratio of UWMS pH. C. Mean (+/-SEM) pH of UWMS and SWMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol in the 18-30-year-old group. D. Mean (+/-SEM) pH of UWMS and SWMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol in the 60+ year old group. Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups). P = < 0.05 * P = < 0.01 ** P= 0.0001 to 0.001*** P= < 0.0001****.

Previous studies have shown an effect of salivary pH on taste sensation. Therefore, pH of WMS was assessed immediately following saliva collection. The mean pH of UWMS was 6.7 ± 0.1, n=14 for the younger and 6.8 ± 0.1, n=11 for the older group (Figure 3-4 A). There were no significant differences in salivary pH of UWMS or SWMS, when normalised to water rinse, between the older and younger groups. The pH of SWMS was not significantly different from water rinse, following any of the taste, TRP or odour stimulations (Figure 3-4 B). The pH of SWMS was significantly higher than UWMS in the younger group for menthol (7.18 +/-0.13), MSG (7.21 +/-0.10) and capsaicin (7.30 +/-0.13) (Figure 3-4 C). In the older group water rinse (7.33 +/-0.08), menthol (7.45 +/-0.05), capsaicin (7.53 +/-0.06), MSG (7.36 +/-0.07) and caffeine (7.37 +/-0.06) stimulated WMS had a significantly greater pH than UWMS (Figure 3-4 D).

Table 3-2 Kendall's tau b correlation between UWMS pH and sensory perception in older (n=10) and younger (n=14) adults. P = < 0.05 * P = < 0.01 ** P = < 0.001 *.**

	Age of Participant			pH resting saliva
Kendall's tau_b	18-30 Years Old	Perception water rinse 1-10	Correlation Coefficient	.024
			Sig. (2-tailed)	.911
			N	14
		Perception menthol odour 1-10	Correlation Coefficient	.296
			Sig. (2-tailed)	.161
			N	14
		Perception menthol 1-10	Correlation Coefficient	-.035
			Sig. (2-tailed)	.867
			N	14
		Perception caffeine 1-10	Correlation Coefficient	-.057
			Sig. (2-tailed)	.782
			N	14
		Perception MSG 1-10	Correlation Coefficient	.035
			Sig. (2-tailed)	.867
			N	14
		Perception capsaicin 1-10	Correlation Coefficient	.049
			Sig. (2-tailed)	.818
			N	14
	60+ Years Old	Perception water rinse 1-10	Correlation Coefficient	-.199
			Sig. (2-tailed)	.452
			N	10
		Perception menthol odour 1-10	Correlation Coefficient	.024
			Sig. (2-tailed)	.927
			N	10
		Perception menthol 1-10	Correlation Coefficient	.047
			Sig. (2-tailed)	.855
			N	10

		Perception caffeine 1-10	Correlation Coefficient	.135
			Sig. (2-tailed)	.590
			N	10
		Perception MSG 1-10	Correlation Coefficient	-.163
			Sig. (2-tailed)	.523
			N	10
		Perception capsaicin 1-10	Correlation Coefficient	.471
			Sig. (2-tailed)	.067
			N	10

To assess the effect of salivary pH on taste, TRP and odour sensitivity, a linear regression (Kendall's tau b's correlation) was conducted between UWMS pH and perception, flow rate, extensional rheology, protein and mucin of UWMS and SWMS. UWMS pH was not significantly correlated with any of the taste, TRP or odour perceptions, although the positive correlation to capsaicin perception was close to significance in older adults (p=0.067) (Table 3-2). There was no significant correlation between UWMS pH and any of the other salivary parameters measured (Appendix 5).

3.3.4 Extensional Rheology of UWMS and SWMS

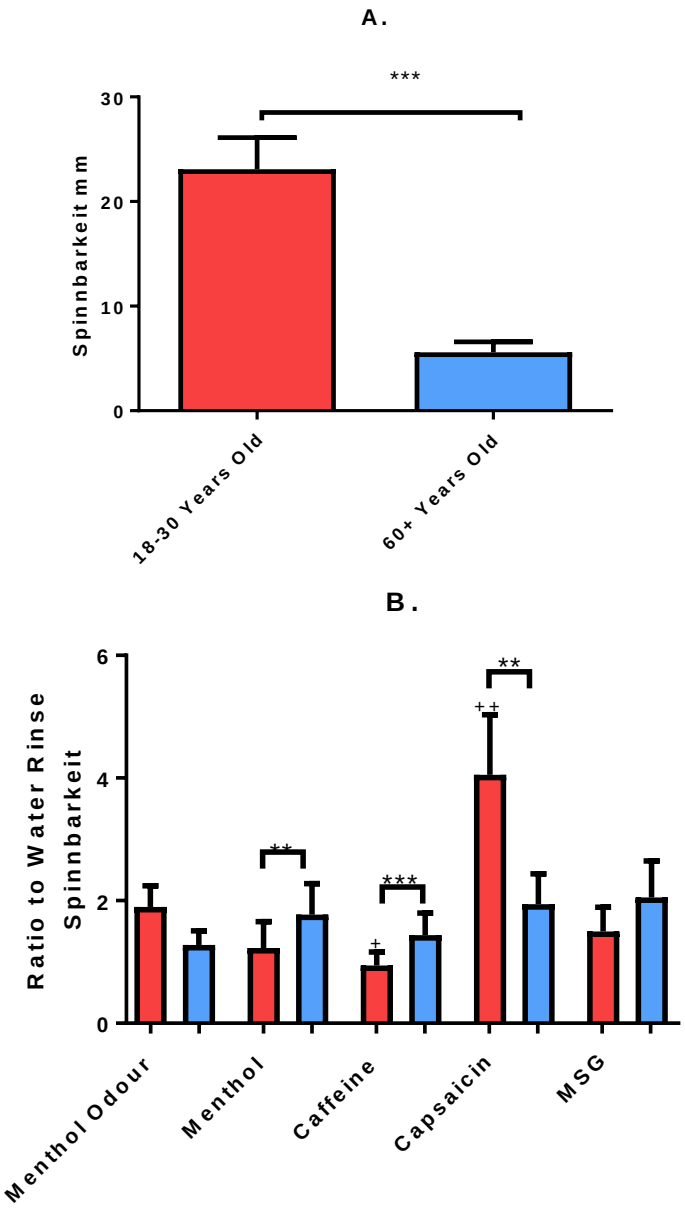


Figure 3-5 Spinnbarkeit of UWMS and SWMS in older and younger adults

A. Mean (+/-SEM) Spinnbarkeit (ER) of UWMS. P = < 0.05 */+ P = < 0.01 **/++ P = < 0.001 ***/+++. B. Mean (+/-SEM) Spinnbarkeit (ER) of WMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol, assessed using the NevaMeter. ER (mm) following taste/TRP stimulation expressed as a ratio of ER (mm) following water rinse. ER (mm) following menthol odour stimulation expressed as a ratio of resting ER (mm). 18-30 years old n=31, 60+ years old n=25. Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups).

Extensional rheology (ER) or Spinnbarkeit gives a measure of the stringiness of saliva, which may be important for transduction of tastants to the taste pore. To investigate whether the physical properties of saliva were altered with age and/or by taste, smell and TRP stimulation, all saliva samples were measured using the NevaMeter for ER. As shown in Figure 3-5 A, the average (+/-SEM) ER of UWMS was significantly greater in the group of younger adults compared to older, 23.09 +/-3.02mm (18-30) and 5.59 +/-0.99mm (60+), indicating an effect of age not only on the quantity of saliva produced but also its physical properties (Figure 3-5 A). Menthol and caffeine mouth rinsing induced significantly lower ER in the younger group, 1.23 +/- 0.43 (Menthol) and 0.94 +/-0.22 (caffeine) compared to 1.78 +/- 0.50 (menthol) and 1.44 +/-0.36 when using ER of WMS following water rinse as a baseline. Capsaicin evoked the highest increase in elasticity in the younger group, significantly greater than baseline, up to 35.26 +/-5.24mm from 17.29 +/-3.42mm (not normalised to water rinse – see appendix 4 for raw data), as well as significantly greater than the older group, 4.05 +/- 0.98 compared to 1.94 +/- 0.49 ratio to water rinse baseline. Neither rinsing with MSG nor smelling of menthol had any significant effect on the ER of WMS (Figure 3-5 B).

3.3.5 Salivary Total Protein Levels

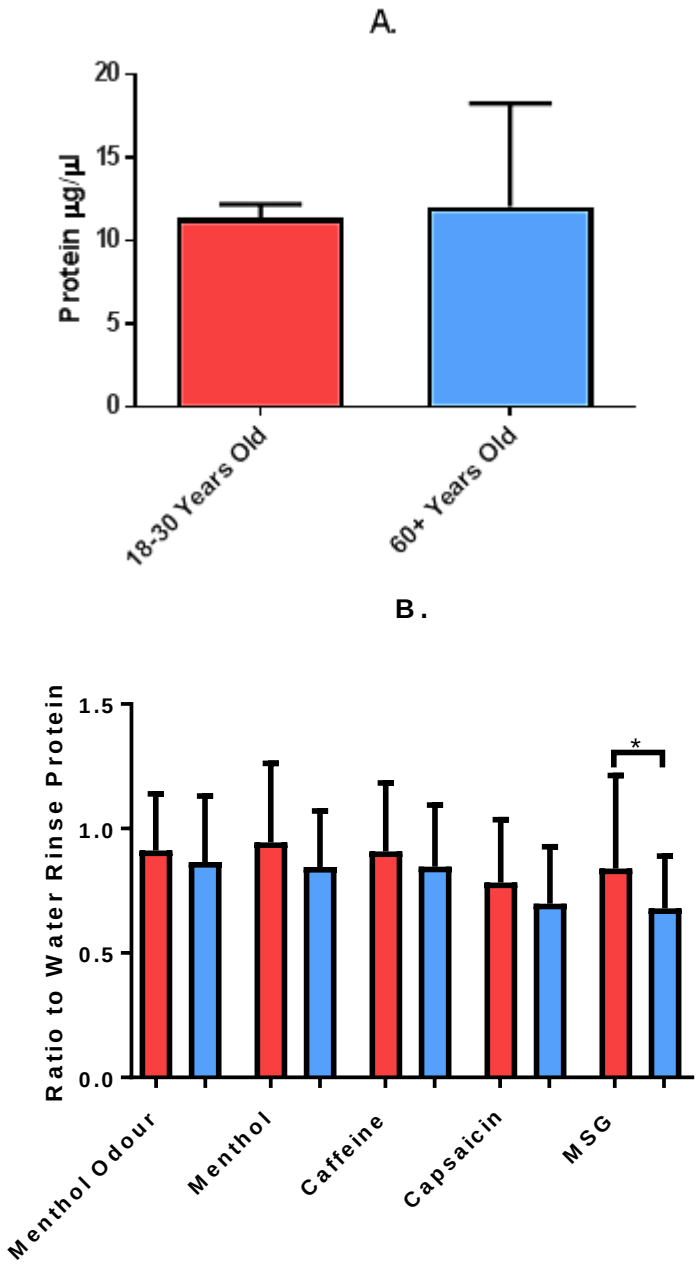


Figure 3-6 Total Protein Levels in UWMS and SWMS in older and younger adults
A. Mean (+/-SEM) total protein of UWMS. P = < 0.05 *. B. Mean (+/-SEM) Total Protein of WMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol, assessed using BCA assay. Total protein (µg/µl) following taste/TRP stimulation expressed as a ratio of total protein (µg/µl)) following water rinse. Total protein (µg/µl) following menthol odour stimulation expressed as a ratio of resting total protein (µg/µl). 18-30 years old (n=31), 60+ years old (n=25). Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups).

Salivary proteins, especially mucins, contribute to the viscoelastic properties of saliva. Since the flow rate and ER of saliva were altered by different stimuli, and appear to be reduced with advanced age, the protein composition of saliva samples was investigated. The average (+/-SEM) total protein content of UWMS was not significantly different between the age groups, 11.35 +/- 0.83 μg/μl (18-30 years) and 12.04 +/- 1.27 μg/μl (over 60 years) (Figure 3-6 A). The SEM for total protein (μg/μl) in unstimulated WMS was greater for the older group suggesting a greater degree of heterogeneity for protein levels in saliva amongst this age group. MSG mouth rinsing induced WMS with significantly higher protein levels in the younger group compared to the older, 0.84 +/- 0.07 (arbitrary values, ratio to water rinse WMS protein) (18-30 years) and 0.68 +/- 0.04 (arbitrary values, ratio to water rinse WMS protein) (60+ years). There was no significant difference between protein levels in WMS whilst smelling of menthol or following mouth rinses with menthol, caffeine and capsaicin between the two age groups (Figure 3-6 B). Additionally, none of the mouth rinses or smelling of menthol, induced WMS with altered total protein level from baseline (water mouth rinse or resting saliva respectively).

3.3.6 Carbonic Anhydrase VI

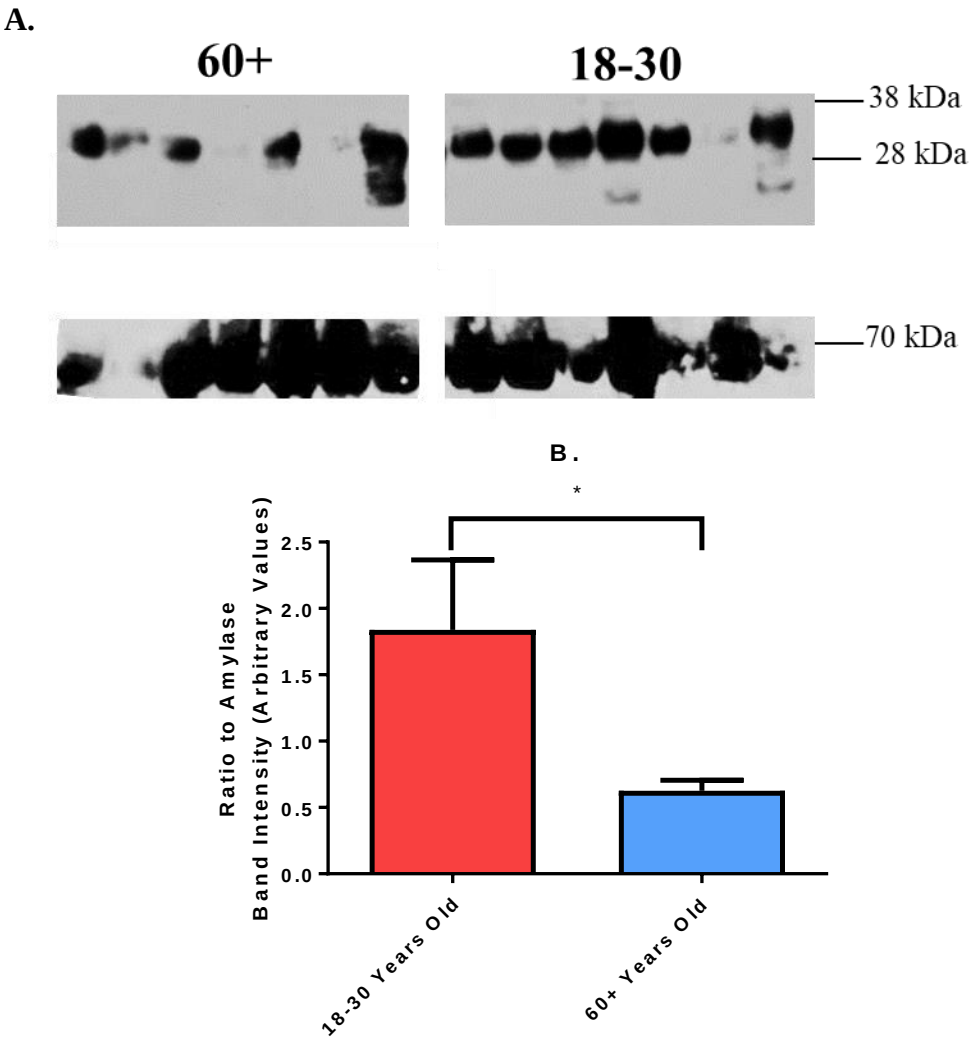


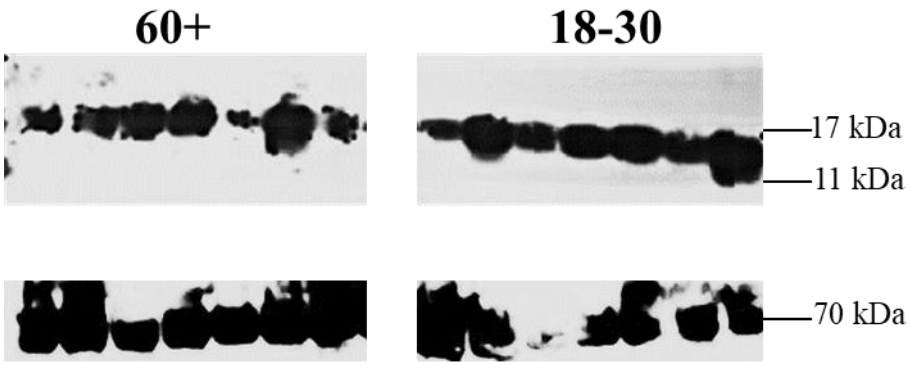
Figure 3-7 Carbonic Anhydrase VI in UWMS of older and younger adults

A. Representative image of carbonic anhydrase VI in UWMS of younger (18-30 years old) and older adults (60+ years old). Expected molecular weight 35kDa. Amylase, used as loading control, expected molecular weight 65kDa. B. Mean (+/-SEM) carbonic anhydrase VI level in UWMS of younger (n=28) and older adults (n=23). Band intensity expressed as a ratio of amylase band intensity to control for loading. Samples with no detectable amylase band were excluded from data analysis. P = < 0.05 *. Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups).

As salivary carbonic anhydrase VI (CAVI) has been associated with taste loss in previous studies, UWMS samples from both age groups were analysed using western blotting for CAVI. The mean (+/-SEM) CAVI in younger UWMS was significantly higher compared to older, 1.838 +/-0.5 and 0.6256 +/-0.1 respectively (Figure 3-7).

3.3.7 Cystatin S

A.



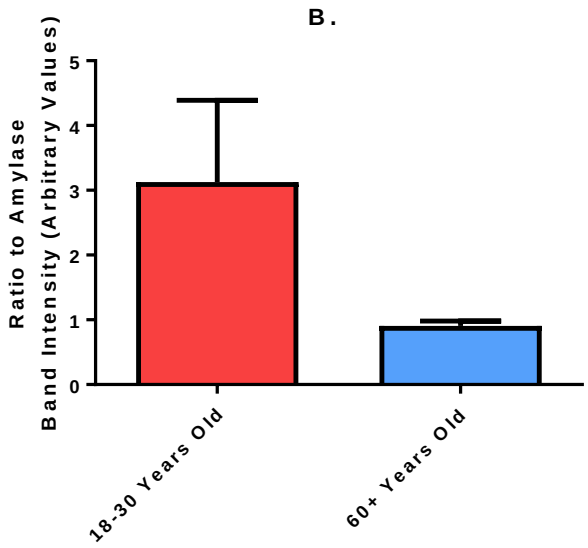
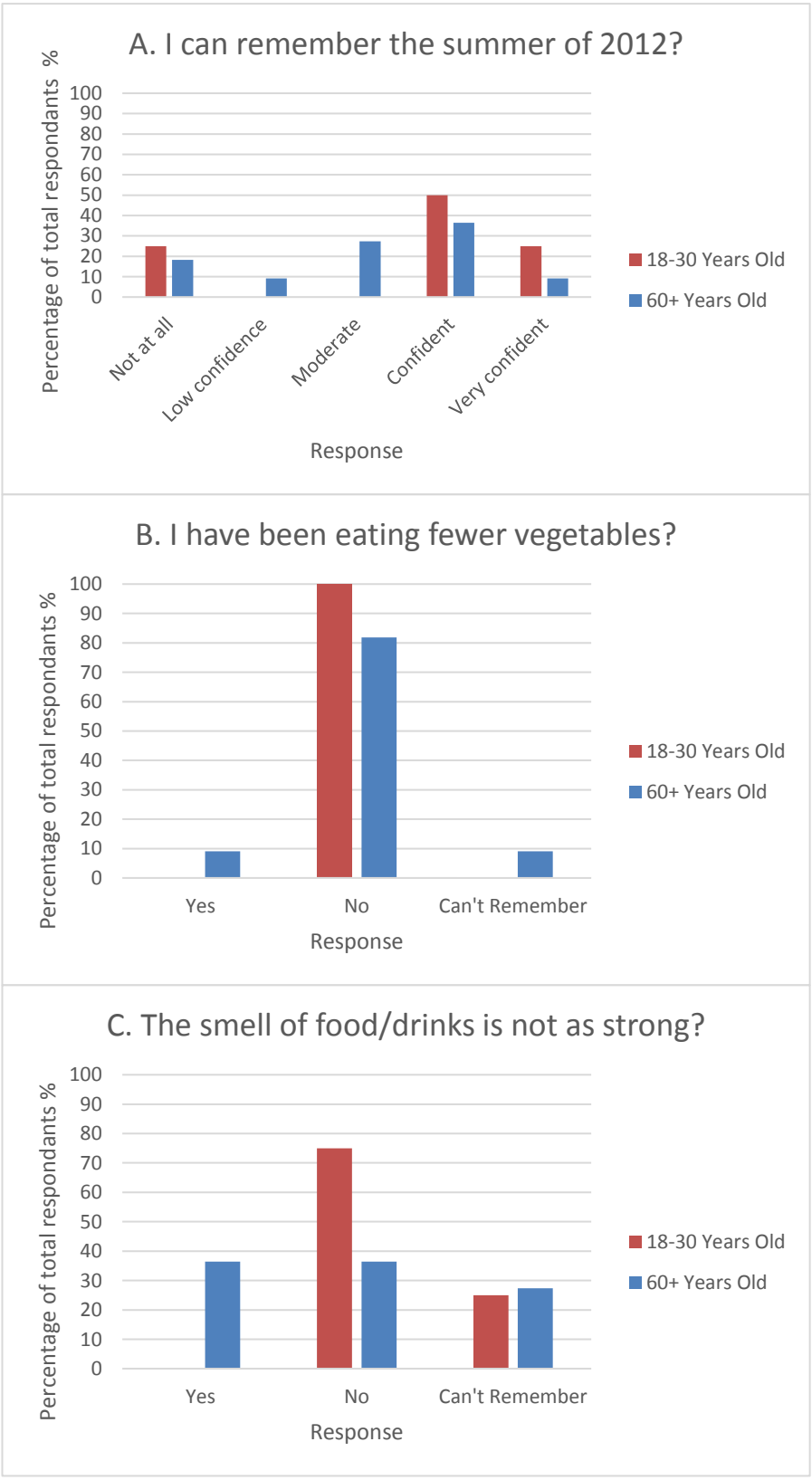


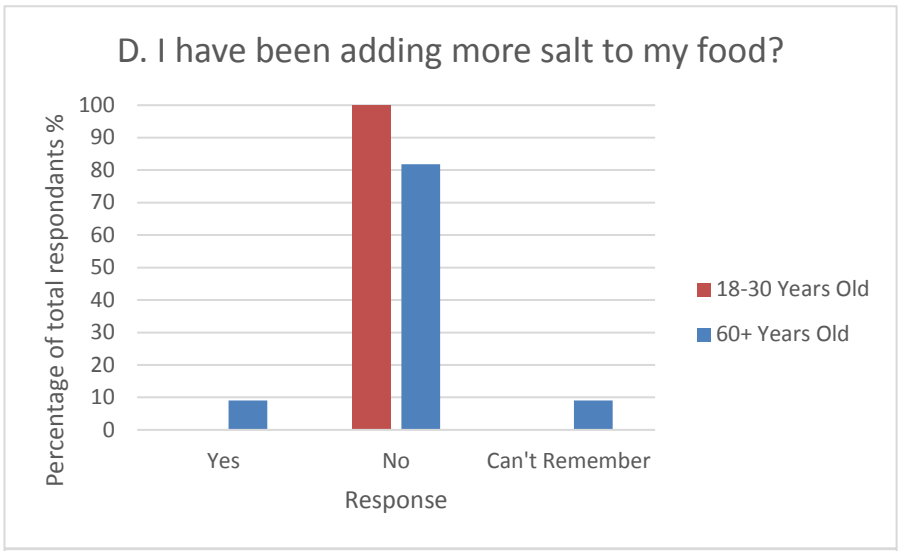
Figure 3-8 Cystatin S in UWMS of older and younger adults

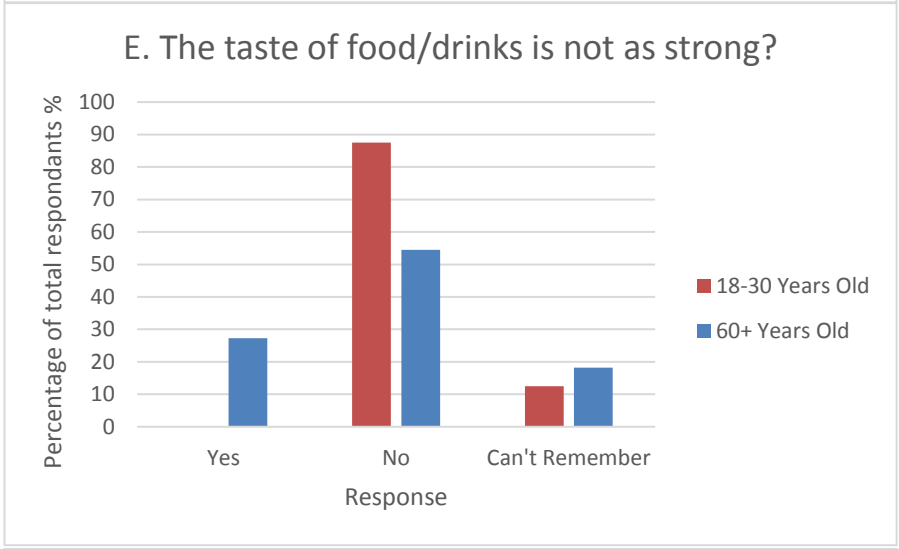
A. Representative image of cystatin S in UWMS of younger (n=30) and older adults (n=25). Lane 1-7 = 60+ years old, 8-14 = 18-30 years old. Expected molecular weight 16kDa. Amylase, used as loading control, expected molecular weight 65kDa. B. Mean (+/-SEM) cystatin S level in UWMS. Band intensity expressed as a ratio of amylase band intensity to control for loading. Samples with no detectable amylase band were excluded from data analysis. Tested for statistical significance using one-way ANOVA (mean difference from baseline within groups) and independent students T-Test (mean difference between groups).

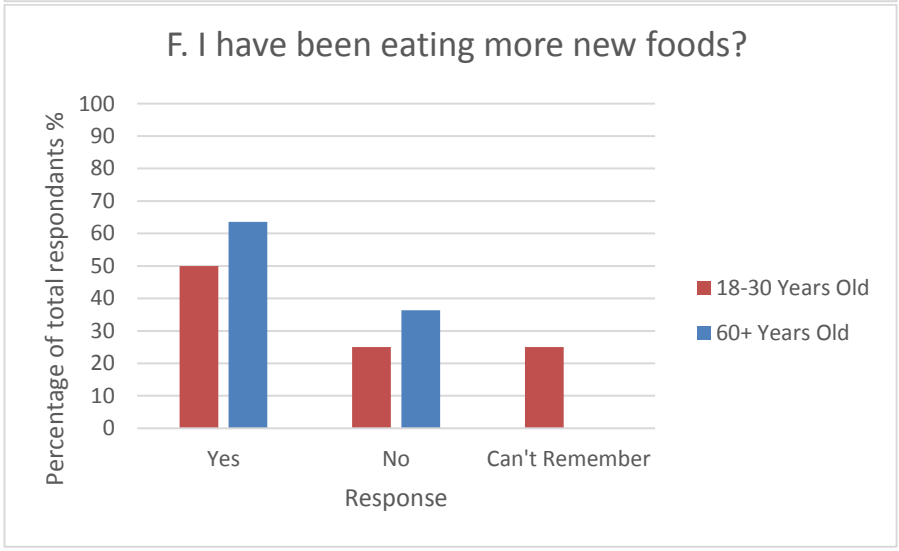
The level of cystatin S in UWMS was not significantly different between age groups. However, there was generally more cystatin S in saliva from the younger group, 2.786 +/- 1.1 compared to 1.102 +/-0.1 in the older group (arbitrary values, Figure 3-8). There was high inter-individual variation in the saliva samples of the younger group, reflected in the high SEM value, which may account for the lack of significance between groups.

3.3.8 Food Behaviours and Self-Reported Taste and Smell Loss




D. I have been adding more salt to my food?
Percentage of total respondants %
100
90
80
70
60
50
40
30
20
10
0
Yes
No
Can't Remember
Response
18-30 Years Old
60+ Years Old


E. The taste of food/drinks is not as strong?
Percentage of total respondants %
100
90
80
70
60
50
40
30
20
10
0
Yes
No
Can't Remember
Response
18-30 Years Old
60+ Years Old


F. I have been eating more new foods?
Percentage of total respondants %
100
90
80
70
60
50
40
30
20
10
0
Yes
No
Can't Remember
Response
18-30 Years Old
60+ Years Old

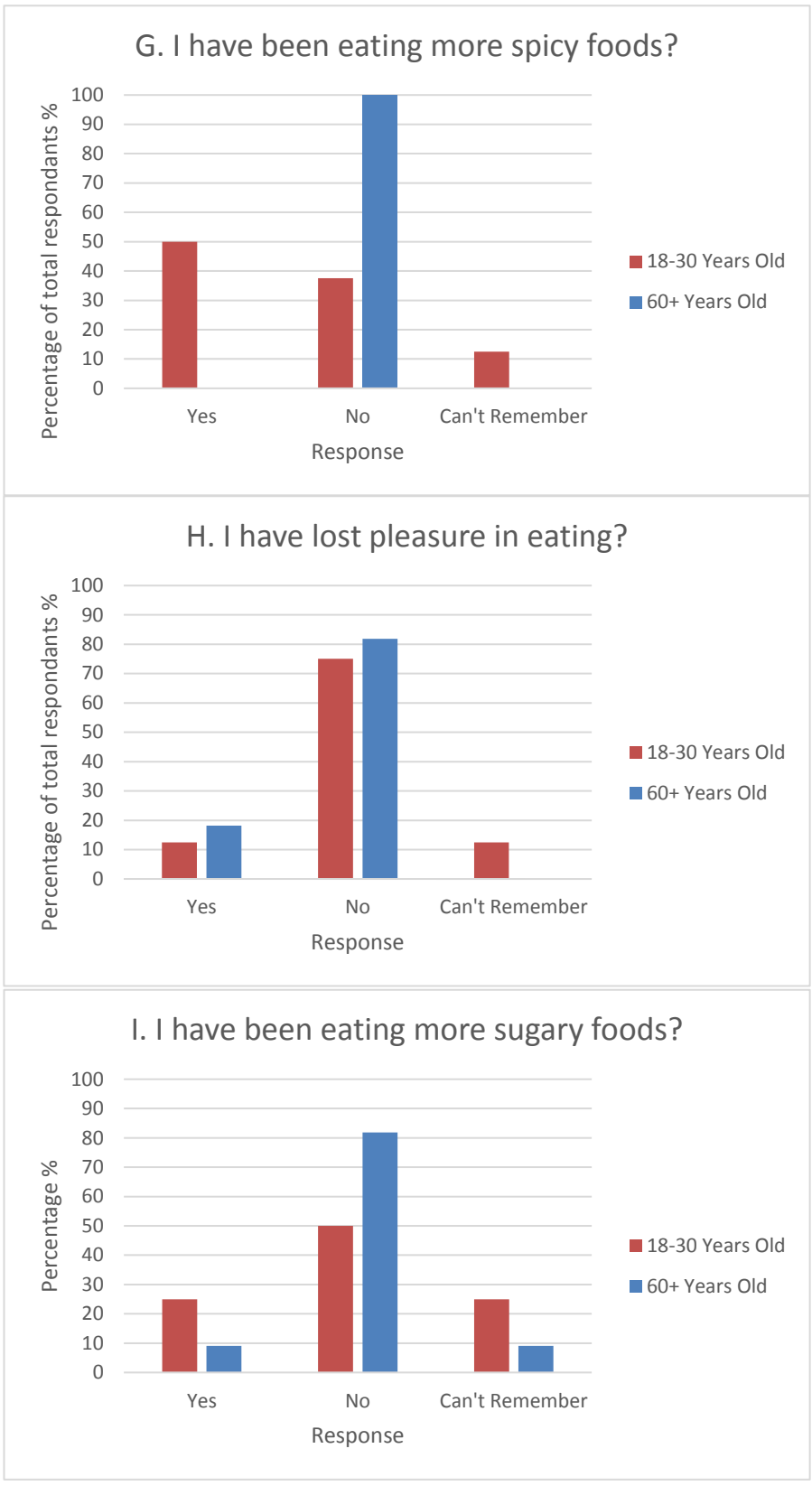


Figure 3-9 Food behaviours and beliefs about taste in older and younger adults
A-I. Responses to questionnaire about food behaviours and beliefs about taste and smell loss. 18-30 years old n=8, 60+ years old n=11. Data shown as percentage of total respondents for each age category.

To uncover individual beliefs about taste and smell loss, a questionnaire was given to participants asking several questions about food behaviours and sensory loss in the past 5 years. Compliance with the questionnaire was low overall, with 44% of the 60+ group and 26% of the 18-30 group completing the questionnaire. The first question asked participants whether they could remember the summer 2012 Olympics, to encourage recall and measure memory of respondents. Most participants in both age groups could remember the event to some extent, 75% of the 18-30 years old group and 81.8% of the 60+ years old group (Figure 3-9 A). There were no significant differences in the responses between age groups. A greater proportion of the older group said they had been eating less vegetables, adding more salt to food, eating more new foods and had lost pleasure in eating within the past 5 years (Figure 3-9 B, D, F, H). More of the younger group said they had been eating more spicy and sugary foods in the past 5 years, compared to the older group (Figure 3-9 G, I). None of the younger respondents said they had experienced taste loss within the 5-year period of recall, while 27.3% of the older group said they had (Figure 3-9 E). Additionally, 36.4% of the older group said they had experienced smell loss in the last 5 years while none of the younger group had (Figure 3-9 C).

Table 3-3 Mean (+/-SEM) Medication Use in Older (N=11) and Younger (N=8) Adults

Medication Type	60+ Years Old	18-30 Years Old
No. Prescribed Meds	1.9 (+/- 0.72)	0.3 (+/- 0.16)
No. over counter meds	1.2 (+/- 0.62)	1.3 (+/- 0.62)
Anti-depressants	0.3 (+/- 0.2)	0
Anti-hypertensives (blood pressure medications)	0.5 (+/- 0.2)	0
Anti-hyperlipidaemic (cholesterol medications)	0.4 (+/- 0.15)	0
Anti-Anxiety	0.1 (+/- 0.1)	0
Narcotics/Painkillers	0.3 (+/- 0.2)	0
Diuretics (water tablets)	0	0
Antacids	0.1 (+/- 0.1)	0

Table 3-3 shows the reported medication use in both age groups. The older age group reported taking an average of 1.9 (+/- 0.72) prescribed medications and 1.2 (+/- 0.62) over the counter medications each, per day. The younger group reported taking an average of 0.3 (+/- 0.16) prescribed medications and 1.3 (+/-0.62) over the counter

medications per person, per day. The differences in medication use were close to statistical significance, p=0.057 and p=0.5590 respectively. Anti-hypertensives were the most common type of medication used by older adults, out of all taste affecting medicines listed on the questionnaire.

Prescription medication use could be positively correlated to spinnbarkeit of MSG stimulated WMS in older adults (Kendall's tau b coefficient 0.744, p=0.003) and negatively correlated to spinnbarkeit of menthol SWMS in the younger group (Kendall's tau b coefficient -0.655, p=0.046) (Appendix 6. Table 9-4). Prescription or over the counter medication use was not correlated to salivary flow rate in either age groups (Appendix 6. Table 9-5). Prescription medication use could be negatively correlated to taste perceptions for MSG in both age groups, Kendall's tau b coefficient: 18-30 years. -0.67, p=0.04, 60+ years. -0.61, p=0.02 (Appendix 6. Table 9-6). There were no other correlations between medication use and perception of taste, TRP or odour stimulants.

3.4 Discussion

3.4.1 Taste, TRP and Odour Responses in Younger and Older Adults

While taste perceptions are subjective and rely on the individual's experiences and environmental factors (Lawless and Heymann 2010), salivary flow occurs as an autonomic reflex response to stimulation and is an objective measure . There was some correlation between taste and smell perception and salivary flow rate as reduced umami taste perception was coupled with a reduced UWMS flow rate in the older group. Capsaicin significantly increased salivary flow compared to water in both age groups, more so in the younger indicative of greater reflex response in the younger participants. Although not statistically significant, there was generally a greater salivary response to all the stimulants in the younger group, even when perceptions were similar between groups, such as for menthol and caffeine. SWMS flow rate may be a more sensitive, objective measure of taste ability than subjective reporting. This method of assessment could prove especially useful in older adults who may have a decline in cognitive function which might otherwise affect accuracy of results from sensory testing.

Only one concentration of each stimulant was tested to allow for testing of a greater range of stimuli without adding to the time burden on participants. Greater differences may have been seen for taste, odour and TRP perceptions and responses if a variety of concentrations and compounds had been used. Previous studies have demonstrated that sensitivity to threshold concentrations of taste stimuli are not always different between older and younger adults (Mojet et al. 2003, Cowart 1989, Weiffenbach et al. 1986a). This may be due to an inverse relationship between concentration and intensity perception for some tastants in individuals with a "normal" sense of taste (Mojet et al. 2005, Webb et al. 2015). Therefore, young adults without taste loss perceive higher concentrations of those tastants as less intense, but older adults experiencing taste loss may not. Caffeine was used as a bitter stimulus in this study and may have an inverse suprathreshold concentration-perception relationship (Keast and Roper 2007). Caffeine has shown to be a concentration dependent inhibitor of IP_3R3, an essential component of the TAS2R signalling pathway (Bezprozvanny, Bezprozvannaya and Ehrlich 1994, Gees et al. 2014). As such, higher concentrations of caffeine may elicit a reduced response compared to lower concentrations, in adults with normal taste sensation. This may be the reason why older adults did not display reduced perception or salivary response to the suprathreshold concentration of caffeine used in this study, despite well

documented deterioration of bitter taste thresholds in ageing (Yoshinaka et al. 2016, Nilsson 1979, Mojet et al. 2003, Mojet et al. 2001, Nordin et al. 2007).

Additionally, sensitivity to suprathreshold concentrations may be more resistant to age related losses than sensitivity to threshold concentrations (Stevens and Cain 1993, Methven et al. 2012). This could be due to threshold concentrations requiring greater sensitivity for detection compared to suprathreshold concentrations. Therefore, the extent of age related taste loss may have been under estimated in this study. Never the less, the assessment of reduced taste sensation at suprathreshold concentrations is of value since detection thresholds are significantly lower than concentrations found in foods. As such, measurement of suprathreshold sensitivity gives insight into sensory loss at a level which may represent eating in real life.

There were more female subjects in both age groups and women may experience age related taste loss with reduced severity compared to men. Several studies have demonstrated that there is no effect of gender on taste function in young subjects, but men are more susceptible to age related decline in taste acuity (Yoshinaka et al. 2016, Wardwell, Chapman-Novakofski and Brewer 2009, Heft and Robinson 2010, Yamauchi, Endo and Yoshimura 2002). In the present study gender had a significant effect on perception of menthol and capsaicin, with females showing greater sensitivity than males (Appendix 7. Table 9-11). No effect of gender was seen on any of the taste or odour stimuli tested here however that may be because the number of male subjects was too low for an effect to be seen.

Mouth rinsing between taste test solutions is a contentious issue in taste testing. There is potential for carry over of tastants if the mouth is not rinsed between different tastes. In line with previously published work showing that mouth rinsing with water, between compounds, did not affect taste thresholds, no mouth rinses were performed during this study (Brosvic and McLaughlin 1989). This is also because rinsing washes away the salivary pellicle and because water may have sialagogic properties which could influence the properties of subsequently expectorated saliva (Houghton et al. 2017). It is thought that mouth rinsing between tests is only required for salt taste which is subject to taste adaptation because of salts present in saliva which perpetuate the salt taste response after stimulation (Yamauchi et al. 2002). To limit carry over of tastant, participants were asked to rest until they could no longer perceive the preceding tastant before presentation of the next stimulus. Additionally, water rinse was presented as the

first stimulant and menthol and capsaicin were presented last, in that order, as these compounds may linger in the mouth and continue to cause stimulation after expectorating.

Increased age was also associated with reduced menthol odour perception. Previous studies also reported lower perceptions in older adults for olfaction, including menthol odour perception (Murphy 1983). Interestingly, there were no differences for perception of oral TRP stimulants, unlike basic taste and smell, so chemo-sensation may not be impaired by age to the same extent as taste and odour. It was expected that capsaicin sensation would be retained in the older age group as this has been shown previously (Fukunaga, Uematsu and Sugimoto 2005a, Forde and Delahunty 2002). However, it has previously been suggested that perceptions of menthol solution administered orally are reduced in older adults (Kremer et al. 2007b, Koskinen et al. 2003). Discrepancies in findings may be due to differences in the testing method employed. It is interesting that menthol odour perception was reduced in the older group while perception of the mouth rinse, which would be perceived by a combination of trigeminal stimulation and retro nasal olfaction, was not reduced. This finding indicates that loss of olfactory function may contribute to reduced perception of menthol in older adults more so than loss of trigeminal sensation. Certain TRP agonists may therefore be useful in improving hedonic aspects of eating and encouraging food intake in older adults with taste loss, as their sensation is not impaired by age.

Even though perception of oral TRP stimuli appeared to be retained with age, the salivary reflex responses to menthol and capsaicin differed between the age groups. Perceptions of TRP stimuli may be affected by the different experiences and environment of older adults while salivary responses are not. It may be that the older generation are less used to or accepting of different sensations in food because certain ingredients were not commonly available until more recently. It is known that childhood eating experiences play an important role in attitudes and behaviours later in life (Edfors and Westergren 2012). This could apply to chilli or capsaicin as well as menthol (derived from mint) as imported herbs and spices are examples of foods which were not readily available due to rationing from 1940 to 1954 in the UK (Foster and Lunn 2007). Previous studies have found that liking of spicy foods is reduced in older adults (Guido et al. 2016). Older people may therefore report that the sensation is stronger than is being sensed physiologically because they are less accepting of oral burning or cooling.

Conversely, SWMS reflex response may be impaired in older adults. Age related reduction in SWMS flow can be linked to an overall reduction in submandibular/sublingual flow. However, age related reduction in UWMS flow is around 66% greater than for stimulated flow, suggesting that the reflex salivary response is somewhat retained compared to resting salivary flow (Affoo et al. 2015). Altered resting salivary composition could also play a role in reduced response to TRP agonists, since, if the quality of older people's saliva is reduced it may transduce the stimulants to the receptors less well than in a younger individual. As such, there may be altered TRP receptor activation in ageing and salivary response is therefore impaired following stimulation.

PTC taste sensation could be correlated with overall taste, TRP and odour sensitivity in younger but not older participants. This suggests that the effect of TAS2R38 polymorphism on overall sensory function is greater in younger adults. Younger adults were more sensitive to PTC than the older group. This contrasts with previous studies which did not find a link between age and PTC taster status (Abraimov and Mirrakhimov 1979, Whissell-Buechy 1990). There are several possible explanations for this. Firstly, this study used filer paper strips instead of PTC solutions. Older adults have reduced salivary flow, and this might affect the way in which PTC is transduced to the taste receptor from a filter paper strip as saliva is required as a dissolution medium. Secondly, previous studies used a series of concentrations of PTC to assess taster status in individuals. This study used one suprathreshold concentration and assessed intensity, but thresholds were not determined. Previous studies using the same method for PTC/PROP taster testing found that suprathreshold sensitivity correlated well with recognition threshold measures (Zhao et al. 2003b, Bartoshuk et al. 1994, Kaminski, Henderson and Drewnowski 2000). However, those studies only looked at young healthy adults, so it is possible that older adults do not display differences to younger adults in threshold for PTC but their sensitivity to suprathreshold concentrations is reduced. Additionally, since genotyping was not conducted in this study it is not possible to determine whether the small sub-group of older adults were genetically pre-disposed to being unable to taste PTC. 25% of the general population are non-tasters with the TAS2R38 AVI polymorphism. Only 9 older adults were included in the PTC test in this study and it could be that many or all of those were PTC non-tasters.

Interestingly, previous studies have shown that individuals with greater sensitivity to PTC also have increased sensitivity to TRP agonists including capsaicin (Bartoshuk et al. 1994, Nolden et al. 2016). The results of this study corroborate that as older adults had lower salivary responses to capsaicin and menthol than younger adults, who also had greater sensitivity to PTC. Perception of PTC could be correlated to SWMS flow rate in response to MSG and caffeine in younger volunteers but not in older (Appendix 8, Table 9-12). Therefore, younger individuals who were more sensitive to PTC also had increased SWMS flow rate in response to the basic tastants tested. It should be noted, that SWMS flow rate in response to water rinsing was also correlated with PTC perception in this age group. However, taste perception for water rinse was not. There are some studies which have shown that water has a perceived bitter or salty taste dependent on mineralisation levels (Teillet et al. 2010, Bruvold and Ongerth 1969). Further, propylene glycol, included in the water rinse since it was used to dissolve some of the stimulants, has a slightly sweet taste (Cruz et al. 2002). Therefore, SWMS following water rinse, may have been due, in part, to stimulation of taste receptors at low levels not usually perceived. That said, the most likely explanation for SWMS resulting from water rinsing is mechanical stimulation due to the action of mouth rinsing. Overall, the results are largely in agreement with previous studies which showed greater sensitivity for bitter and umami tastants in individuals who exhibit greater sensitivity for PTC/PROP (Hong et al. 2005a).

Medication use has previously been reported as a causal factor in age related taste loss (Smith 1994). In this study, a questionnaire was used to gather data on medication use in older and younger adults. The sample size was unfortunately low due to low compliance with the questionnaire, however older adults had greater medication use on average compared to younger adults. Medication use was negatively correlated with MSG taste perception in both age groups suggesting that use of medication may be linked to reduced MSG taste sensation. Furthermore, use of certain medication has also been linked to reduced salivary flow (Ichikawa et al. 2011). In this study there were no correlations between medication use and salivary flow. This may be because a larger sample size would be needed to establish a link between medication use and salivary flow. Alternatively, because this study recruited exclusively healthy older adults, their medication use may have been lower than the older adult population on the whole.

3.4.2 Physical Properties of Saliva in Younger and Older Adults

Since saliva is required for normal taste function, reduced flow rate and altered physical properties might play a role in impaired taste sensation. Saliva with high viscoelasticity is likely to be highly muco-adhesive and therefore provide improved surface wetting and protection of oral surfaces including the tongue. Additionally, saliva is a solvent in which tastants are solubilized to be transported to taste receptors. In the present study it was demonstrated that flow rate and viscoelasticity of saliva may reduce with age as ER and flow of resting saliva was significantly greater in the younger subjects compared with older. It could be hypothesized therefore that older adults with reduced salivary ER might also have reduced taste sensation because of poor muco-adhesion of saliva on the oral epithelium which may mean saliva cannot transduce or solubilize tastants as well. A reduced flow rate could also affect taste function since less saliva present in the mouth may mean oral surfaces less well protected by the salivary layer, perhaps leading to atrophy of taste cells. These findings contrast with the results shown by Zussman et al. and Kazakov et al, suggesting that saliva from older people had increased viscoelasticity ((Kazakov et al. 2009) Zussman et al. 2007). This may be due to differences in testing conditions as both previous studies measured relaxation times using uni-axial elongational flow or tensiometry while in the present study extensional viscoelasticity was measured as Spinnbarkeit or stringiness. Relaxation times of saliva were shown to increase over time following expectoration of saliva samples which was suggested to be due to aggregation of mucin, however, extensional viscoelasticity is depleted over time (Kazakov et al. 2009, Wagner and McKinley 2017). This indicates that the two methods do not measure the same property and thus, the results are not comparable.

Previous studies measuring Spinnbarkeit using the NevaMeter method have shown ER of healthy UWMS to be anywhere from 44.0 ± 5.9 mm to 28.5 +/- 5.7 mm, while for xerostomic patients this value drops to 9.2 +/- 4.3 mm, (Houghton et al. 2017, Chaudhury et al. 2015). This suggests that the younger group were within the range of "healthy" while the older group had similar ER range to xerostomic patients. This is probably due to the submandibular/sublingual salivary flow being subject to the greatest age-related reduction while parotid salivary flow, which does not contain mucin, is unaffected by age. This means that the saliva of an older individual contains a greater proportion of parotid saliva (Affoo et al. 2015). It is known that parotid saliva has no

mucin content and therefore also has a significantly lower viscoelasticity compared to submandibular/sublingual saliva (Zussman et al. 2007, Vijay et al. 2015). Previously, it has been shown that Spinnbarkeit can be a sensitive measure for identifying Sjögren's syndrome as it is reduced even in cases where the patient suffering dry mouth has a normal flow rate (Chaudhury et al. 2016). Along with the results presented here, this shows that Spinnbarkeit can be an important measure of the "quality" of saliva which may have equal importance as flow or "quantity" of saliva.

In a recent study from our group (Houghton et al. 2017) it was hypothesized that TRP stimulants may be useful in relieving dry mouth in xerostomic patients. The present results support the idea that TRP agonists, particularly capsaicin, could stimulate secretion of WMS with greater muco-adhesion and viscoelasticity which might reduce feelings of dry mouth. However, this may only be useful in young patients, as neither of the TRP agonists tested here caused saliva secretion with increased ER in the older participants. Houghton et al. (2017) also demonstrated that menthol evoked WMS had increased ER, which was not the case here. The difference in results may be because the previous study had only 6 participants, therefore inter-individual variation could have greater effect on the results. Additionally, WMS was collected in the first minute after stimulation separately from the second minute. Any increase in ER following menthol stimulation was only seen during the first minute and therefore was said to be an instant and transient effect. It was suggested that mucin stores could have been depleted following the initial stimulation of saliva secretion so that WMS expectorated subsequently had lower ER. In the present study, saliva was collected for 2 minutes after stimulation so the initial ER stimulating effect of menthol may have been partially masked.

As mentioned above, there was a larger proportion of female subjects here and women generally have lower salivary flow rates than men (Percival, Challacombe and Marsh 1994). Yet, the age-related decline in salivary flow is proportional between genders (Affoo et al. 2015). Hormones such as estrogen can influence salivary composition and females have smaller salivary glands which may affect saliva secretion and composition, however this would be mostly applicable to the younger subject group or to pre-menopausal females (De Almeida et al. 2008). Females also have lower salivary pH and reduced buffering capacity, total protein, MUC5B and secretory IgA levels but increased MUC7 and lysozyme activity compared to males (Prodan et al. 2015).

However, in the present study, there was no significant effect of gender on UWMS or SWMS flow rates (Appendix 7. Table 9-10). There was however a significant effect of gender on total protein content of UWMS, which was reduced in females compared to males (Appendix 7. Table 9-8). Also, spinnbarkeit was significantly greater in UWMS and menthol and caffeine SWMS of female subjects compared to male (Appendix 7. Table 9-9). There was no significant effect of gender on any of the other salivary parameters tested (Appendix 7. Table 9-8). It is important to note however that these results should be interpreted with caution given the limited number of male subjects included in the statistical analysis for gender effects (n=10). A larger proportion of male subjects would be required to draw substantial conclusions about the effect of gender on the parameters studied in this thesis.

3.4.3 Salivary Chemical Properties and Composition in Younger and Older Adults

pH of unstimulated saliva has been reported to be around 6.6 +/-1.23 (Vijay et al. 2015). The present study found pH of UWMS to be 6.7 ± 0.1 in the younger and 6.8 ± 0.1 for the older group, in agreement with the literature. A higher pH has previously been linked to heightened sweet taste sensitivity, however a correlation was only apparent in around 38% of tested individuals with the highest and lowest pH values (Matsuo and Yamamoto 1990, Aoyama et al. 2017). In this study, increased salivary pH could not be correlated with perception of any taste, TRP or odour stimulant. Thus, pH of saliva may be related specifically to sweet taste sensation and not to other basic tastes. pH of saliva may affect physical properties such as viscoelasticity since an increased pH has previously been linked to reduced salivary viscoelasticity (Vijay et al. 2015). However, salivary pH could not be correlated to spinnbarkeit in this study.

Previous studies also showed an increase in salivary pH following stimulation due to increased bicarbonate from a greater proportion of parotid saliva in SWMS compared to UWMS (Gittings et al. 2015, Vijay et al. 2015, Bardow et al. 2000). The pH values for MSG taste stimulated saliva agree with previously reported values by Vijay et al who showed a pH of 7.2 +/-1.67 (Vijay et al. 2015). In this study, pH of MSG SWMS was 7.21 +/-0.10 in younger adults and 7.36 +/-0.07 in older adults. There were significant differences in salivary pH following stimulation with menthol, capsaicin and MSG compared to UWMS in younger adults (Appendix 9. Table 9-15). In older adults all taste and TRP SWMS had a greater pH than UWMS (Appendix 9. Table 9-14). As previously discussed, there may be a greater proportion of parotid saliva in SWMS of

older adults compared to younger (Affoo et al. 2015). Salivary pH is altered as the fluid flows through the salivary gland ductal system (Humphrey and Williamson 2001). At first it is an isotonic fluid released in the secretory acinus and then sodium and chloride ions are absorbed, and secretion of bicarbonate and potassium leads to a hypotonic fluid being secreted from the duct. Each acinus contains either solely serous cells, solely mucous cells or both (Proctor 2016). The parotid gland is made up of mainly serous cells which secrete a fluid rich in electrolytes including bicarbonate (Humphrey and Williamson 2001). Therefore, parotid saliva contains higher levels of bicarbonate than saliva from other glandular sources, which may explain why the SWMS pH values were generally higher in older adults. Even water rinsing gave rise to an increase WMS pH in older adults, suggesting the stimulation of the parotid gland occurring was not solely due to taste or TRP receptor activation and suggesting a contribution of mechanical stimulation occurring from mouth rinsing. This is further corroborated by the fact that menthol odour led to the lowest pH of all stimulants tested, in older adults, and was the only stimuli which did not involve a mechanical rinsing action. Furthermore, previous studies have shown that, unlike the submandibular/sublingual glands, secretion of the parotid gland is not increased by smell stimulation (Lee and Linden 1992). On the other hand, mechanical stimulation such as chewing does preferentially stimulate the parotid gland (Stokes and Davies 2007).

Levels of carbonic anhydrase VI (CAVI) and cystatin S were reduced in older adults compared to younger. This was only statistically significant for CAVI, perhaps due to high inter-individual variation in cystatin S levels in the younger group. Reduced CAVI has previously been linked to taste loss since individuals with taste loss of multiple aetiologies were shown to have reduced salivary CAVI (Shatzman and Henkin 1981, Igarashi et al. 2008). Furthermore, salivary CAVI has been shown to be lower in PROP non-tasters compared to tasters and positively correlated to taste perceptions (Rodrigues et al. 2017, Walliczek-Dworschak et al. 2017). It is thought that salivary CAVI may function as a growth factor to promote regeneration of taste cells (Thatcher et al. 1998). Some studies have found that polymorphisms in the CAVI gene can lead to reduced zinc binding and affect the density, development, and maintenance of foliate taste papillae (Padiglia et al. 2010, Calò et al. 2011, Melis et al. 2013b) but others have found no such association (Feeney and Hayes 2014). As such, further investigation is required to understand the mechanism behind CAVI in taste function and whether reduced levels in older people's saliva could be contributing to reduced sensitivity to taste. It is also

possible that older adults are more prone to zinc deficiency since this has previously been correlated to reduced salivary CAVI (Komai et al. 2000). Breifel et al reported that 66% of adults over 70 years old had inadequate zinc intake (Briefel et al. 2000). Furthermore, older adults suffering from xerostomia also have significantly lower zinc intakes compared to non-xerostomic older adults (Rhodus and Brown 1990). Thus, zinc deficiency may be linked with reduced salivary CAVI observed here in older adults and in reduced salivary flow rates.

Conversely, previous studies have shown that reduced cystatin S in saliva is associated with heightened sensitivity to bitter tastants (Dsamou et al. 2012, Morzel et al. 2014). This may be because cystatin S acts as a protease inhibitor, reducing proteolysis in saliva which leads to a thicker layer of saliva in the mouth that may act as a barrier to some tastants. However while proteolytic activity was shown to be increased in individuals with reduced salivary cystatin S, this was not statistically significant and the rheology of saliva was not tested (Dsamou et al. 2012). Furthermore, this was only shown in infants and in young healthy adults, therefore the role of cystatin S in taste function of older adults has not been demonstrated. Previous studies correlated cystatin S to detection thresholds, but no association was shown to sensitivity to suprathreshold concentrations as used in the present study. It is possible therefore that reduced proteolysis because of increased cystatin S in saliva may lead to reduced threshold detection but not suprathreshold sensitivity. In agreement with the findings from this study, reduced S-like cystatins (S and SN) has been shown in saliva of patients with taste dysfunction, and interestingly the mean age of the patient group was 59.5+/- 6.1 years compared to a mean age of 27.8+/- 2.5 years in the healthy group (Igarashi et al. 2008). Also, low sensitivity to sweet taste compounds has been correlated to reduced salivary cystatins (Rodrigues et al. 2017). It could be hypothesised that a thicker saliva is better retained in the oral cavity and thus retention of taste compounds is increased with a resulting enhanced sensitivity. The idea of increased proteolytic activity in saliva of older adults, because of reduced cystatin S, is interesting since saliva from the older group did have reduced viscoelasticity and reduced MUC7 levels. As such, degradation of mucin was studied in further detail in the subsequent chapter of this study.

Also, certain proteins which share homology with the cystatin family have shown to enhance sweet taste by binding to the sweet taste receptor and stabilising the active form in a “wedge” mechanism (Tancredi et al. 2004, Temussi 2011). Therefore, it is possible

that cystatins can enhance sensitivity to certain tastants by stabilisation of the active form of the receptor.

3.4.4 Food Behaviours and Self-Reported Sensory Loss in Older and Younger Adults

Compliance with the sensory loss questionnaire was low overall and as such it was not possible to draw significant conclusions from the results. None of the younger adults disclosed loss of smell or taste in the past 5 years but a proportion of older adults did. Furthermore, compared to younger respondents, a greater number of older adults said they add more salt to their food and have lost pleasure in eating, in the last 5-years. In general, older adults are prone to minimising health attributes which may be considered negative due to social desirability bias (Nordin et al. 1995). Social desirability bias may be greater in older adults due to a desire to dispel negative stereotypes of ageing (Dijkstra et al. 2001). Therefore, the results may be under-reporting age related changes in eating habits and sensory loss.

Further, when asking people to recall past behaviours there is a danger that they will have forgotten and may therefore make up responses to please the researcher (Wagenaar 1986, Asimakopoulou and Hampson 2005). In this study, a question was included to see if participants felt they could remember a significant event from 5 years ago, the London 2012 Olympics. This then acted as a reference point from which subsequent questions could be related to. However, some participants said they could not remember that event at all and others were not confident they could remember. Previous studies have shown that recall ability is related to significance of the event, and so an event was chosen that would have significance to most people who were living in London at that time (Coughlin 1990). However, significance of the chosen event to each individual is still likely to vary. Additionally, there was some difficulty in selecting a significant event which would have the same significance to individuals in both age groups.

Additionally, asking about food behaviour and sensory loss in the past 5 years may not have captured the point at which age-related taste loss would occur since it may begin as early as middle age (Green et al. 2013). However, choosing an event more than 5 years ago may have negatively impacted the ability for subjects to recall (Wagenaar 1986). Therefore, the results of the questionnaire should be interpreted with caution and

may not reflect actual levels of dietary changes and sensory loss within the older population.

Some studies asking adults, who completed food intake diaries 10 years ago or more, to recall food intakes from that period, showed that the ability to recall general dietary habits, such as major food groups, was relatively accurate (Eysteinsdottir et al. 2011, Sobell et al. 1989). However, recollection of more detailed food intake information, such as intake of individual foods, was not well correlated with the intake diaries from the past (Eysteinsdottir et al. 2011). In this study questions referred to intakes of major food types such as sugar or salt and therefore, remembering of this kind of information may be relatively accurate.

Interestingly, none of the older respondents said they ate more spicy foods in the past 5 years while 50% of the younger respondents said they had. This is in line with previous studies which found that older adults are less accepting of spicy foods (Guido et al. 2016). As such, this may be a reason why the objective salivary responses had greater difference between older and younger adults compared to the subjective perceptions for capsaicin. These results highlight the usefulness of salivary responses to oral stimulants as an objective measure of sensitivity which is not subject to environmental factors or subject bias.

3.5 Concluding Remarks

Taken together, these results show the impact of age upon taste and smell sensation, while trigeminal sensations may be less affected by ageing. These changes in sensation may also lead to reduced salivary response to taste, TRP and odour stimulation in older adults. This is because these stimuli can cause altered physical and compositional properties of saliva as a reflex response in young healthy adults. As such, measurement of salivary flow rate and even extensional rheology could be a useful tool for sensory testing as it is not affected by individual experience and cognitive factors which can impact upon sensory perceptions. Salivary flow and elasticity is reduced in older adults and this may play a role in impaired taste function. It was therefore proposed that levels of MUC7 and MUC5B may be reduced in UWMS of older adults, contributing to reduced viscoelasticity and possibly causing reduced muco-adhesion of saliva in this age group. Since muco-adhesion may impact upon taste function and the ability of saliva to transduce tastants, the second part of the study was directed towards characterisation of salivary rheology in relation to mucin characteristics and muco-adhesion of UWMS in older adults. This aimed to further characterise which physical properties of saliva are important for taste function and may be altered in the older population.

4 Characterisation of Physical Properties of Saliva in Ageing: Muco-Adhesion and Rheology

N.B. I would like to acknowledge the contribution of Professor Pietro Cicuta and Nicola Pellicciotta (Cavendish Laboratory, Department of Physics, Cambridge University) who kindly provided advice and assistance in using the dynamic differential microscopy (DDM) technique used in this chapter. All the practical DDM experimental work was conducted at the Cavendish Laboratory by myself under the supervision of Nicola Pellicciotta. Subsequent image analysis was conducted by Nicola Pellicciotta who calculated the viscosity values which were analysed statistically by myself.

4.1 Introduction

*In this study the terms viscoelasticity, spinnbarkeit and extensional rheology are used interchangeably but all refer to the stringiness of saliva.

Mucins are salivary proteins responsible for viscoelastic, muco-adhesive properties of saliva (Van der Reijden, Veerman and Nieuw Amerongen 1994). The results from chapter 3 demonstrated an age-related reduction in salivary viscoelasticity*, which may result from altered mucin composition in saliva from older adults. There are two major salivary mucins, MUC5B and MUC7, both highly glycosylated, that form non-covalent bonds to create a gel like network (Inoue et al. 2008). Salivary mucins, MUC5B and MUC7 are heavily o-glycosylated with an extended linear structure consisting of peptide backbones, rich in serine, threonine and proline residues. While Ser and Thr are substrates for O-linked glycosylation, Pro residues contribute to maintaining the polypeptide backbone in an extended conformation (Coles, Chang and Zauscher 2010, Bansil and Turner 2018). The higher molecular weight MUC5B has a central core containing seven cysteine rich domains (Davies et al. 2016). These highly conserved, hydrophobic “CYS domains”, are involved in intradomain disulphide bonding, and also mucin-mucin hydrophobic interactions, important for the structure of the mucin gel network (Ambort et al. 2011). Mucin monomers are linked by inter-chain disulphide bonds formed by cysteine residues present in cysteine “knot” domains at the carboxy-terminal region, and form dimers which then further polymerise to form multimers (Bansil and Turner 2018). The structure of the mucin gel is stabilised by formation of hydrophobic and electrostatic bonds, facilitated by mucin-mucin interactions and sialic

acid residues (Demouveaux et al. 2017). The hydrophilic glycan chains of mucins allow for hydration of the mucin gel, through interaction with water molecules (Bansil and Turner 2018). MUC7 has a single glycosylated region and smaller non-glycosylated domains which impart greater hydration and solubility compared to MUC5B (Mehrotra, Thornton and Sheehan 1998). MUC7 is incorporated into the mucosal pellicle through formation of non-covalent complexes with other proteins and mucin-mucin interactions with MUC5B (Gibbins et al. 2014, Gibbins et al. 2015).

Previous studies have shown reduced MUC7 in saliva from older adults, using glycoprotein staining of SDS PAGE gel electrophoresis of saliva (Denny et al. 1991, Navazesh et al. 1992). Glycoprotein staining, such as PAS used in this study and stains-all used in previous studies, show glycosylated forms of mucin, therefore de-glycosylation of mucin due to degradation, would show as reduced levels when quantifying using this method (Baughan et al. 2000). As such, it has not been possible to elucidate whether salivary mucin secretion is reduced in older adults or if there is a greater level of oral mucin degradation, occurring in ageing. For this reason, in this study, PAS staining of glycoproteins was compared with immuno-blotting of mucin peptide cores, to investigate de-glycosylation of salivary mucin in older adults.

Sialidases (neuraminidases), sulfatases and α-fucosidases initiate mucin degradation by removing the sialic acids, sulfate, and α-fucose groups from the ends of oligosaccharide chains (Derrien et al. 2010). Glyosidic enzymes expressed by certain oral bacteria, including *streptococci*, then facilitate cleavage of carbohydrate side chains of mucin (Van der Hoeven and Camp 1991). Finally, proteases can digest the exposed peptide back-bone (McGuckin et al. 2011). Several bacteria species express some or all of the enzymes needed for mucin metabolism including, *Streptococcus, Helicobacter, Akkermansia, Bacteroides, Bifidobacterium, Clostridium, Prevotella, Ruminococcus* and *Streptomyces* (Derrien et al. 2010). Oral biofilm bacteria use MUC5B as a food source, degrading the terminal saccharide moieties (Wickström and Svensäter 2008). Also, in dry mouth and Sjögren's syndrome patients, mucin levels in saliva are not significantly reduced but glycosylation of MUC7 is reduced, contributing to reduced hydration of mucin in saliva and feelings of oral dryness in patients (Chaudhury et al. 2016, Chaudhury et al. 2015). As it remained to be seen whether salivary apo-mucin synthesis is reduced or whether MUC7 glycosylation is altered in older adults, western blotting was conducted. Antibodies specific for the peptide backbone of MUC5B and

MUC7 were used, to show relative amounts of the protein core regardless of degradation.

Older adults have high levels of oral bacteria including *Streptococcus mutans, Streptococcus sobrinus* and *Lactobacilli*, as well as yeast in the oral cavity, accompanied by poorer oral health compared to younger adults (Köhler and Persson 1991, Hunt, Drake and Beck 1992, Griffin et al. 2012, Petersen and Yamamoto 2005, Katoh et al. 1997). Indeed, increased salivary *Streptococcus mutans* has been correlated with reduced MUC7 glycosylation in saliva of older adults (Baughan et al. 2000). Additionally, sialidase enzymes are mainly produced by S*treptococci* bacteria (Byers et al. 2000). Therefore, it was hypothesised that salivary mucin in older adults may have greater degradation than in younger adults, thus explaining the reduced PAS staining of SDS-PAGE electrophoresed saliva from older subjects.

Sialidase activity is likely to be the first step in the sequential degradation of mucins since removal of sialic acids exposes the N-terminal region of the peptide backbone to proteolytic activity (Bradshaw et al. 1994, Takehara et al. 2013). Indeed, proteolytic degradation of the peptide cores of MUC7 and MUC5B has shown to be rapidly increased following sialidase treatment (Takehara et al. 2013). Hydroxyl groups on mucin sialic acid residues are highly substituted by acetyl esters (Hutchins and Reading 1988). O-acetylation of sialic acid inhibits their cleavage by glycosidic enzymes and thus is protective against degradation (Corfield et al. 1992). This indicates a protective function of mucin sialic acid residues against proteolysis. However, several bacterial strains also produce O-acetyl-esterases which reduce the protective effect and expose sialic acids to sialidase activity (Corfield et al. 1992).

There are 2 types of sialidase enzymes, trans and exo, which share similar active site residues including tyrosine which acts as the nucleophilic catalyst and aspartate or glutamate residues which provide the acid/base required for the reaction (Damager et al. 2008, Morley et al. 2009). Hydrolysis of terminal sialic acid residues from the glycoprotein occurs through a double displacement mechanism, catalysed by an acid/base from aspartate/glutamate and involving a covalent sialyl–enzyme intermediate (Mehr and Withers 2015). The cleaved sialic acid is transferred to an alternative sugar acceptor (trans-sialidases) or in the case of exo-sialidases, to a water molecule (Amaya et al. 2004, Damager et al. 2008). To further investigate degradation of mucin, lectin blotting was used to semi-quantify the amount of sialic acid in saliva from both age

groups. Detection of α-2, 6 linked sialic acid residues was conducted using Sambucus nigra agglutinin (SNA) lectin and for α-2, 3 linked sialic acid, Maackia amurensis leukoagglutinin II (MAL II) lectin was used. MUC5B has been shown to preferentially bind SNA lectins and MUC7 to preferentially bind MAL II lectins (Chaudhury et al. 2016), therefore these were used to detect sialylation of both salivary mucins in the context of ageing. Removal of sialic acid residues from salivary mucin in older adults could lead to increased perception of oral dryness, because of increased mucin degradation, and reduced functionality of salivary mucin which impairs formation of the visco-elastic mucin gel. In Sjögren's syndrome patients, de-sialylation as well as de-glycosylation of salivary mucin has been shown and this was proposed to be a contributing factor in perceived oral dryness (Chaudhury et al. 2016).

Oral mucins play an important role in protection against pathogenic microbial invasion of the oral cavity (Prakobphol et al. 1999, Everest-Dass et al. 2012, Tabak et al. 1982). MUC7 is capable of agglutinating certain oral bacteria to prevent binding of harmful pathogens to the oral mucosa (Levine et al. 1978). Previously, it has been shown that sialidase treatment of MUC7 reduced its anti-bacterial action, demonstrating the negative effect of sialic acid degradation on oral health (Bucki et al. 2008). This may in turn impact upon taste ability since oral surfaces which are not protected by salivary proteins, such as mucin, may be subject to microbial attack. Furthermore, MUC7 is thought to be responsible for the visco-elastic properties of saliva (Inoue et al. 2008). De-sialylation of salivary mucin leads to reduced viscoelasticity, demonstrating the importance of mucin O-glycan structures for their rheological properties (Ito et al. 1988). As such, de-sialylation of MUC7 could lead to poor lubrication and adherence of saliva to the surfaces of the mouth and tongue which might affect how saliva transduces tastants.

An adsorbed layer of saliva covers all oral surfaces including the tongue and is termed the mucosal pellicle (Bradway et al. 1989). Similarly to the enamel pellicle which covers the surfaces of the teeth, the mucosal pellicle provides lubrication and protection to the soft surfaces in the oral cavity (Bradway et al. 1989, Tabak 2006). Muco-adhesion may also affect the ability of saliva to transduce tastants to the taste buds (Cook et al. 2017). Highly muco-adhesive saliva may enhance tastant dissolution and increase retention on the tongue to potentiate taste sensation (Bradley et al. 2003, Cook et al. 2017). It was therefore hypothesised that saliva from older adults may have

reduced muco-adhesive qualities and therefore reduced retention on oral surfaces, which may be a mechanism behind reduced responsiveness to taste seen in older adults.

To test this theory, an *in vitro* model for muco-adhesion was used. Oral epithelial cells, TR146, were cultured as a confluent monolayer to represent the oral mucosal surfaces. TR146 cells, originating from a squamous cell carcinoma of the buccal mucosa, have previously been used as an *in vitro* model of the buccal mucosa (Jacobsen et al. 1995). The oral cavity is covered by keratinised and non-keratinised stratified squamous epithelium (Squier and Kremer 2001). TR146 cells are a useful tool for drug permeability studies and muco-adhesion since they facilitate mucin adhesion *in vitro*, similarly to the oral epithelium *in vivo* (Teubl et al. 2013).

MUC1 is a membrane associated glycoprotein, expressed in most epithelial tissues, including the oral epithelium (Hanisch and Müller 2000, Chang et al. 2011). Surface bound mucin may facilitate adherence of the mucosal pellicle via mucin-mucin interactions (Gibbins et al. 2015). Previously it has been shown that surface expressed MUC1 is important for muco-adhesion of saliva since it is capable of binding to salivary MUC5B, providing an "anchor" for adherence of the salivary pellicle (Ployon et al. 2016). Additionally, MUC1 expression is reduced in the oral mucosa of older adults, resulting in impaired mucosal defences (Chang et al. 2011). Therefore, this study used TR146 cells which stably over-express MUC1 as a model for muco-adhesion mimicking the environment of the oral cavity. Also, a tongue epithelium cell line, SCC090, was tested for adherence of a salivary pellicle to demonstrate usefulness of this cell line as a model of the tongue epithelium. Saliva samples collected, as described in chapter 3, were used to create a salivary layer on top of the TR146 cells and this was washed away before the cells were lysed and analysed for surface bound mucin to gain insight into changes in surface adherence of salivary mucin in older adults.

Saliva is highly visco-elastic and has a complex surface rheology as well as bulk rheology, namely, viscosity. These physical properties may be important for the functional capacity of saliva such as, solubilising and carrying taste compounds to the taste receptor cells. In chapter 3, it was demonstrated that saliva from older adults had reduced viscoelasticity. However, viscosity may also affect dissolution of tastants since a highly viscous saliva may act as a barrier to taste compounds. Previous studies have shown that high viscosity can lead to reduced solubility of hydrophobic compounds which may be relevant for certain tastants such as some bitter compounds and

sweeteners (Braun and Parrott 1972). Alternatively, saliva with low viscosity could cause reduced taste transduction if thin watery saliva is easily washed away from oral surfaces including the tongue. Salivary viscosity varies in the literature largely due to different measurement techniques employed and different shear rates used (Table 4-1 (Schipper, Silletti and Vingerhoeds 2007)). There are very few studies which have shown the effect of ageing on salivary viscosity. Briedis et al (1980) did not find a difference in viscosity of stimulated saliva between older and younger adults (Briedis et al. 1980). As such, viscosity of UWMS from both age groups was also measured to allow comparison with the viscoelasticity results (described in chapter 3).

Traditional methods for measuring salivary viscosity require relatively large volumes of saliva and are problematic due to the high surface elasticity which may affect measurements (Waterman et al. 1988). Since some older adults had very low flow rates and therefore, limited sample volumes, this study employed a novel method for measuring viscosity of saliva using volumes of less than 50µl. Dynamic differential microscopy (DDM) is a technique which has recently been applied to analysing the viscosity of complex biological fluids (Cerbino and Cicuta 2017). It can measure the dynamic viscosity of a fluid by tracking the movement of fluorescently labelled colloidal particles, interspersed in the sample of interest. Thousands of particles can be viewed at once and as such, data obtained is highly statistically significant (Cerbino and Cicuta 2017). The procedure is simple and cost effective and could provide a standardised technique for measuring viscosity of biological fluids, on a micro scale.

In DDM, the movement of particles is reduced in a more viscous fluid, while in a less viscous fluid, the particles move more freely. The viscosity of a fluid can be calculated using the (constant) slope of the mean squared displacement and the Stokes–Einstein calculation which links the diffusion coefficient, D, of a bead with radius r to the viscosity of the liquid, η, at a given temperature, kBT (Mason et al. 1997). In a purely viscous solution, the mean squared displacement of the particle increases linearly with time (Martin, Forstner and Käs 2002). Particle tracking micro rheology (PTM) has successfully been employed to study the dynamic viscosity of mucus, including purified salivary mucins, but to date there are no such studies using particle tracking to measure viscosity of whole saliva (Georgiades et al. 2014, Davies et al. 2014, Su et al. 2018, Lieleg et al. 2010). Also, traditional PTM, utilises microscope video tracking, but DDM can acquire additional information about the structure and dynamics of a fluid. This is

because it utilises the principles of Fourier optics, to interpret microscopy images, giving results that are equivalent to those obtained using the complex dynamic light scattering (DLS) technique (Cerbino and Trappe 2008). DLS and DDM can be used to study the dynamic properties of biological fluids in situ, for example in living macrophage and red blood cells using the angular and time dependence of scattered light intensity (Amin et al. 2007, Dzakpasu and Axelrod 2004).

The inter-molecular mucin spacing within a mucin gel is around 300 nm, and therefore to determine bulk rheology using PTM, probe particles must be larger than this (Lai et al. 2009). Use of smaller particles will result in diffusion of particles through pores in the mucin gel and thus, the measurement will be affected by the "micro-rheology" within pores. Indeed, the viscosity is around 100 times lower within mucin pores, compared to the bulk viscosity of a mucin gel, because of water molecules interspersed within the mucin gel (Celli et al., 2005). Therefore, to study the bulk viscosity of UWMS, the particles used for viscosity measurements, in this study, were 1000nm in size. Also, binding of polymeric particles used in PTM, to mucin may lead to overestimation of viscosity, as the movement of particles is hindered by mucin-interaction (Lai et al. 2007). Therefore, coating of the beads in a surfactant may eliminate polymer-mucin interaction (Lai et al. 2007). Although, in concentrated mucin gels it has been shown that polyethylene glycol (PEG) coated particles do not behave differently to uncoated particles (Georgiades et al. 2014), this may not be true for more dilute mucin gels (Lieleg et al. 2010) such as those found in saliva. For this reason, in this study, particles used for DDM were coated in (PEG).

Table 4-1 Reported Viscosity of UWMS (Adapted from (Schipper et al. 2007))

A Viscosity (mPas)	Shear rate (s^{-1})	Method	References
2.5, 100	95; 35 °C, 0.02; 35 °C	Low shear rheometer (Contraves)	(Vissink et al. 1984)

A Viscosity (mPas)	Shear rate (s^{-1})	Method	References
4.2	20; 37 °C	Low shear rheometer (Contraves)	(Veerman, Valentijn-Benz and AV 1989)
1.5–1.6	1–300; 37 °C	Oscillating capillary rheometer (Vilastic)	(Van der Reijden et al. 1993)
1.6–1.8	1–300; 23 °C	Oscillating capillary rheometer (Vilastic)	(Van der Reijden et al. 1994)
1.1	70 Hz; 25 °C	Couette-type (Contraves)	(Waterman et al. 1988)
3.8–8.8 (mean 5.7)	90; 37 °C	Cone plate viscometer (Brookfield)	(Rantonen and Meurman 1998)
1.3	26 °C	Capillary rheometer (Cannon)	(Nordbö, Darwish and Bhatnagar 1984)

In chapter 3, taste, TRP and odour responses were characterised in older and younger adults. The results demonstrated changes in saliva due to ageing which may affect taste function, with reduced viscoelasticity in saliva of older adults. The present chapter aimed to further investigate these changes, specifically looking at the mechanism behind alterations in the physical properties of saliva, which may impact diffusion of tastants to the taste buds. Further, this chapter aimed to understand how age affects the mucin composition and rheology of saliva and how this might impact binding of the salivary pellicle to the oral mucosa. Since viscosity of saliva may also affect diffusion of tastants, DDM was employed to measure viscosity of small volume saliva samples to see whether bulk rheology of saliva is affected by ageing. Also, degradation of salivary mucin was assessed using immuno-blotting with anti-bodies directed to the peptide core of MUC7 and MUC5B. This allowed for comparisons to be made between levels of glycosylated forms of mucin (measured using PAS staining) and total amount of mucin

protein in saliva from older and younger adults. Additionally, since sialylation of salivary mucin may affect its functional properties, this was determined using immuno-blot for lectins which preferentially bind MUC7 and MUC5B sialic acids. Finally, a model of the oral epithelium was used to assess muco-adhesive capacity of salivary mucin from both age groups, *in vitro*.

4.2 Methods and Materials

4.2.1 Differential Dynamic Microscopy (DDM)

For measurement of viscosity the un-stimulated WMS saliva samples collected and frozen in chapter 3 were used. Details of the DDM technique can be found in chapter 2.

4.2.2 SDS-PAGE Gel Electrophoresis and Immuno-Blotting

Un-stimulated and stimulated WMS samples were used for further analysis of mucin content. Samples were clarified by centrifugation at 2000G for 10 minutes at 4°C before use in downstream applications. SDS-PAGE was used to separate protein in saliva samples. A control sample of un-stimulated whole mouth saliva (UWMS) from one healthy donor (aged 27 years) was included in each gel to allow for normalizing between gels. Periodic Acid Schiff (PAS) glycoprotein stain was used to detect high molecular weight glycoproteins (MUC5B and MUC7). To identify mucins (immuno-blot), after separation, proteins were transferred by electrophoresis to nitrocellulose membranes. Antibodies used, to detect MUC5B and MUC7 in UWMS, were directed to the peptide core of the mucins (Rousseau et al. 2003).

Membranes were blocked in TBS-T (for MUC5B), 2% (w/v) skimmed milk powder (Marvell, Premier Foods, London, UK) TBS-T solution (for MUC7) or 5% Ovalbumin (Sigma, Dorset, UK) TBS-T (for MUC1) for 1 hour. Membranes were probed using monoclonal antibodies: EU-MUC5Bb 1:500 or EU-MUC7a 1:500 (Novus Biologicals, Abingdon, UK) in TBS-T or 2% skim milk in TBS-T, or in polyclonal MUC1 (Genetex International Corporation, Irvine, CA 92606 USA) in TBS-T. Beta actin monoclonal antibody was used as a loading control, 1:5000 in TBS-T (Thermo Fisher Scientific, Paisley, UK). The blots were exposed to x-ray film, as described in chapter 2, to detect signal.

4.2.3 Detection of Sialic Acid

Detection of sialic acid residues in UWMS was conducted using lectins as previously described (Chaudhury et al. 2016). Briefly, saliva was electrophoresed by SDS-PAGE and proteins were transferred to nitrocellulose membranes, as described in chapter 2, before being probed using biotinylated Sambucus nigra agglutinin (SNA, 0.05 μg/ml), to detect α-2, 6 linked sialic acid, and biotinylated Maackia amurensis leukoagglutinin II (MAL II, 0.4 μg/ml), for α-2, 3 linked sialic acid (Vector Laboratories, CA, USA).

4.2.4 Semi-Quantification of Mucin/Sialic Acid

Densitometry was conducted to semi-quantify band intensities of mucin and lectin blots. Western blot films were scanned, and images were imported into Image J version 1.46 (NIH, MD, USA) for semi-quantification of the band pixel intensity.

4.2.5 Mucin Muco-Adhesion Assay

Binding of salivary mucin, to oral epithelial cells - TR146, TR146 MUC1 and SCC090 - was assessed using the muco-adhesion assay, described in chapter 2.

4.2.6 Statistics

Microsoft Excel (Version 1804, Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA) was used for data analysis and generation of graphs. SPSS version 24 was also used for statistical analysis (IBM Analytics, Armonk, NY, USA). An independent student's t test was used for difference between groups. The student's t test was selected despite lack of normal distribution since there was a great enough subject number to allow use of the parametric test (Lumley et al. 2002). Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****. Bi-variate linear regression analysis was conducted for correlations between salivary parameters and sensation. The Kendall's tau b technique was used to calculate a correlation coefficient and a coefficient of 0 regarded as no correlation, a coefficient of 1 regarded as a positive correlation and a coefficient of -1 as a negative correlation.

4.3 Results

UWMS samples from older and younger adults used in this chapter were the same as those collected and used for analysis in chapter 3.

4.3.1 Assessment of the Dynamic Viscosity of UWMS in Older and Younger Adults

As reduced viscoelasticity of older adults' UWMS was demonstrated in chapter 3, viscosity of UWMS, from both age groups, was also measured to determine the effect of ageing on bulk rheology of saliva. The dynamic viscosity of UWMS was not significantly different between the age groups. However, the younger group did have slightly higher viscosity, 0.00173 +/-0.0002 Pa.s compared to 0.00155 +/- 0.0002 Pa.s in the older group (Figure 4-1).

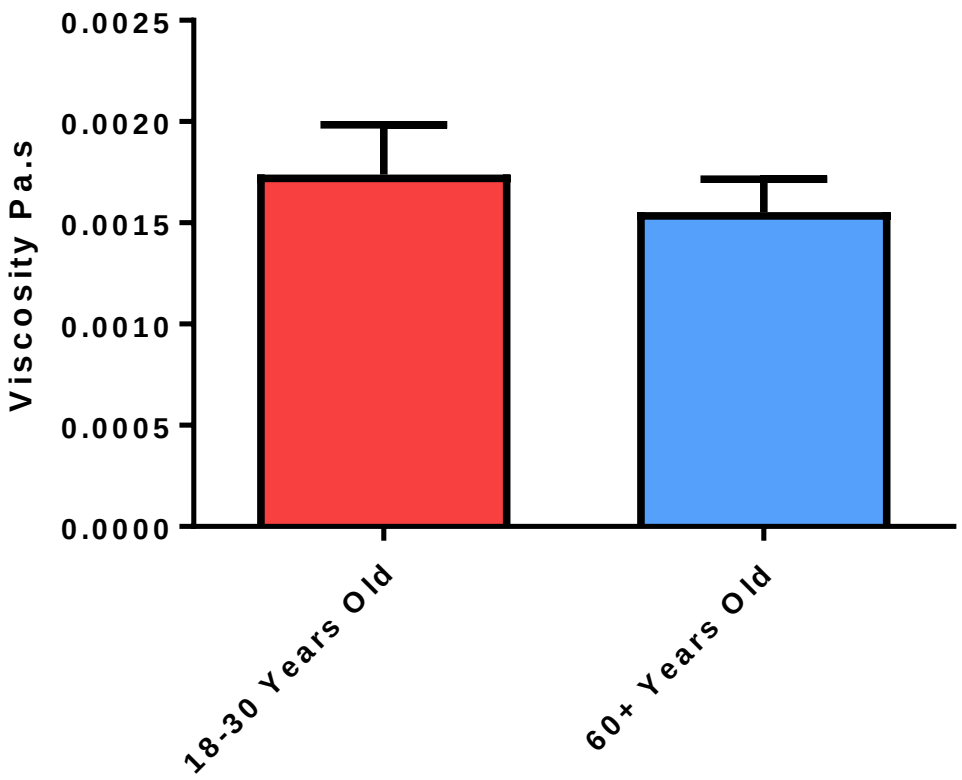
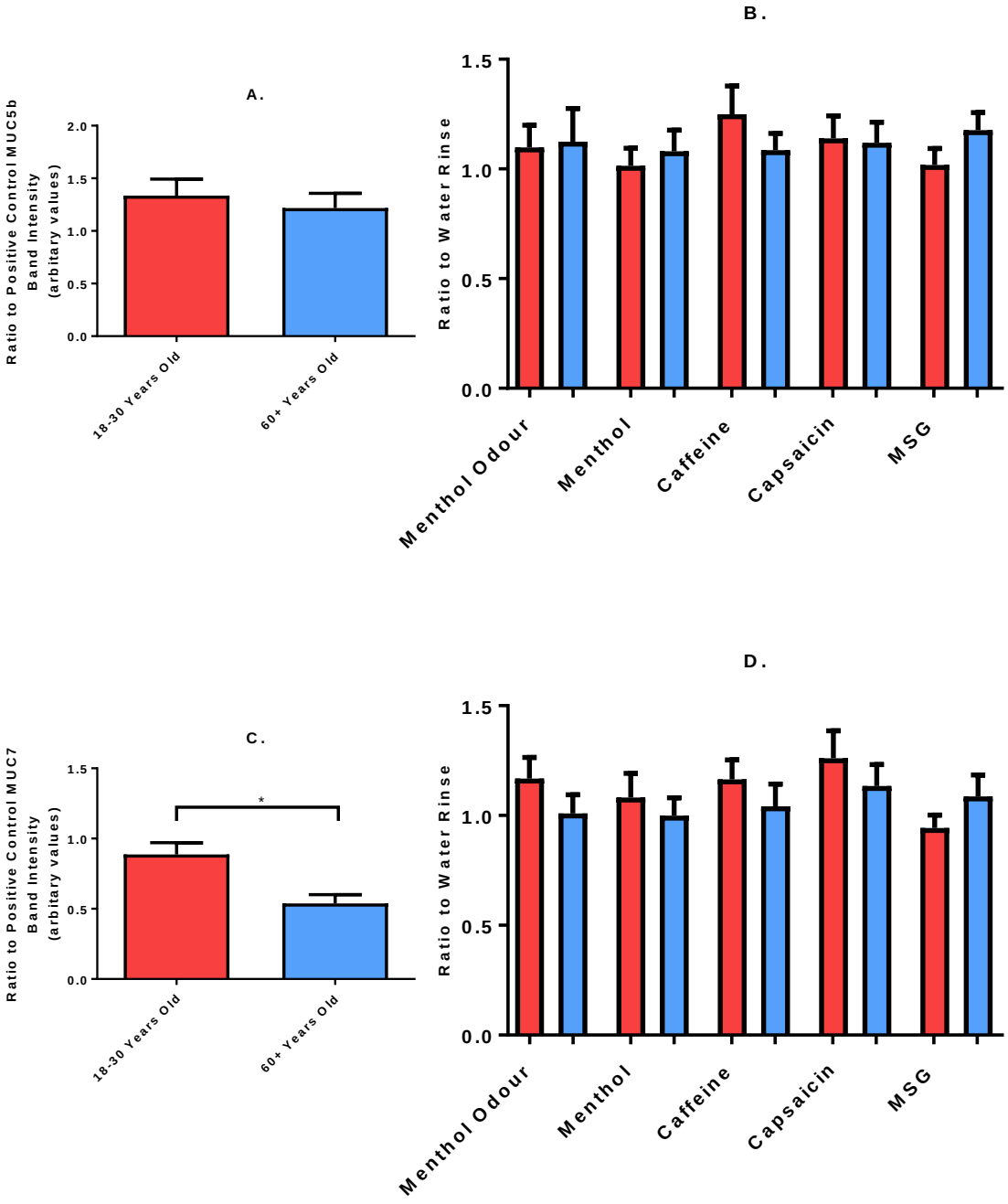


Figure 4-1 Mean (+/- SEM) dynamic viscosity of UWMS from older (N=24) and younger (N=30) subjects, determined using DDM.

4.3.2 Salivary Mucin Levels



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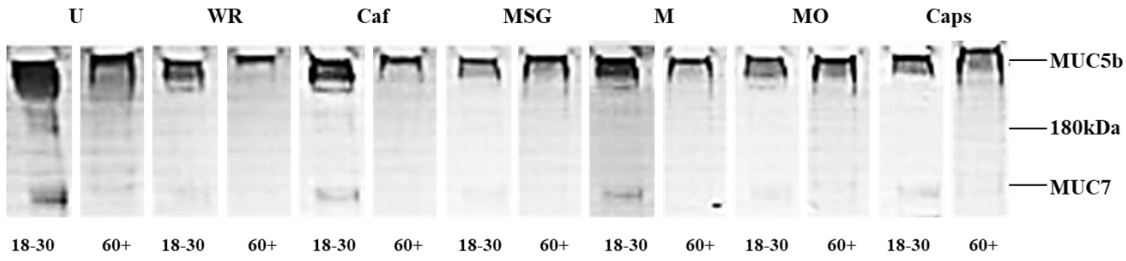


Figure 4-2 MUC5B and MUC7 in UWMS and SWMS of older and younger adults determined by PAS staining of SDS PAGE gel

A. Mean (+/- SEM) MUC5B level in UWMS of older (N=25) and younger (N=31) adults. B. Mean (+/- SEM) MUC5B level in stimulated WMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol, calculated as band intensity on a PAS stained SDS-PAGE gel. C. Mean (+/- SEM) MUC7 level in UWMS. D. Mean (+/- SEM) MUC7 level in stimulated WMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol, calculated as band intensity on a PAS stained SDS-PAGE gel. E. Representative image of an SDS-PAGE gel PAS stained for mucin glycoproteins. R=Resting, WR=Water Rinse, Caf=Caffeine, MSG=Mono Sodium Glutamate, M=Menthol, MO=Menthol Odour, Caps=Capsaicin. Mucin band intensity following taste/TRP stimulation expressed as a ratio of mucin band intensity following water rinse. Mucin band intensity following menthol odour stimulation expressed as a ratio of unstimulated mucin band intensity. P = < 0.05 *. Tested for statistical significance using independent students T-Test.

In chapter 3, it was demonstrated that extensional rheology was affected by age, therefore, further analyses of mucins were carried out, as proteins responsible for rheological properties of saliva. Salivary mucins, especially MUC7, are largely responsible for the ER of WMS. As the ER was different between age groups and dependent on stimulus, the relative levels of mucins, MUC5B and MUC7, were semi quantified using PAS staining of SDS-PAGE gels to see if age or stimulant caused secretion of altered levels of mucin which could explain the altered ER (Figure 4-2). The average level of MUC5B in USWMS or SWMS was not different between age groups (Figure 4-2 A), however there were significantly higher amounts of MUC7 in the UWMS of the younger group compared to the older, 0.89 +/-0.08 compared to 0.54 +/-0.06 (band intensity measured using ImageJ software, arbitrary values) which may explain the higher ER of younger resting WMS (Figure 4-2 C). None of the taste or TRP mouth rinses or the menthol smelling evoked WMS with MUC5B levels that differed significantly from baseline (Figure 4-2 B). However, the MUC7 level in capsaicin stimulated saliva from the younger group was the highest compared to baseline out of all the stimulated WMS tested, on average 26.1% (+/-12.51%) greater (not statistically significant) (Figure 4-2 D). As such, the increased ER in capsaicin stimulated WMS in the younger group may partly be due to increased secretion of MUC7.

4.3.3 Semi-Quantification of Salivary Mucin Using Immuno-Blot in UWMS of Older and Younger Adults

To determine whether expression of MUC7 is reduced or if MUC7 is de-glycosylated in UWMS of older adults, immuno-blotting of mucins was conducted with anti-bodies directed to the peptide core of MUC7 and MUC5B. In agreement with the data from PAS staining (shown above), mean (+/-SEM) levels of MUC7 were significantly higher in UWMS from younger adults compared to older, 1.90 +/- 0.37 and 0.453 +/- 0.15 respectively, P=<001 (Figure 4-3 A). There was no significant difference in MUC5B from UWMS of either age group, when semi-quantified from immuno-blotting, mean ratio to control WMS of 6.02 +/- 0.77 in the younger group and 5.328 +/- 0.99 in the older group (Figure 4-3 A). To allow comparison of mucin measured using anti-bodies directed to the peptide core to PAS staining of glycoprotein, the band intensities were expressed as a ratio to a sample of the same control WMS from one donor, which was run on every gel/blot. There was a significantly higher ratio of MUC7 measured using immuno-blotting of the peptide core compared to PAS staining of in UWMS from younger adults compared to older adults (Figure 4-3 C). There were no significant differences between age groups for the ratio of MUC5B measured by immuno-blot compared to PAS staining (Figure 4-3 C).

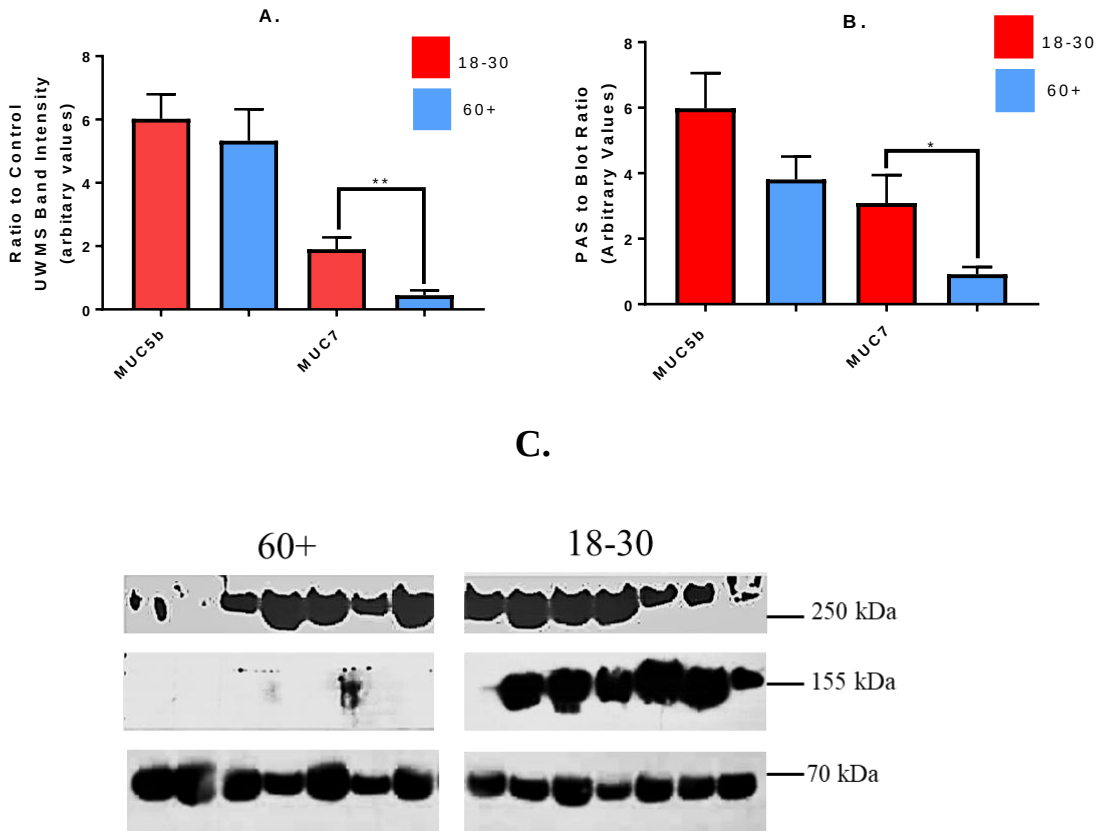
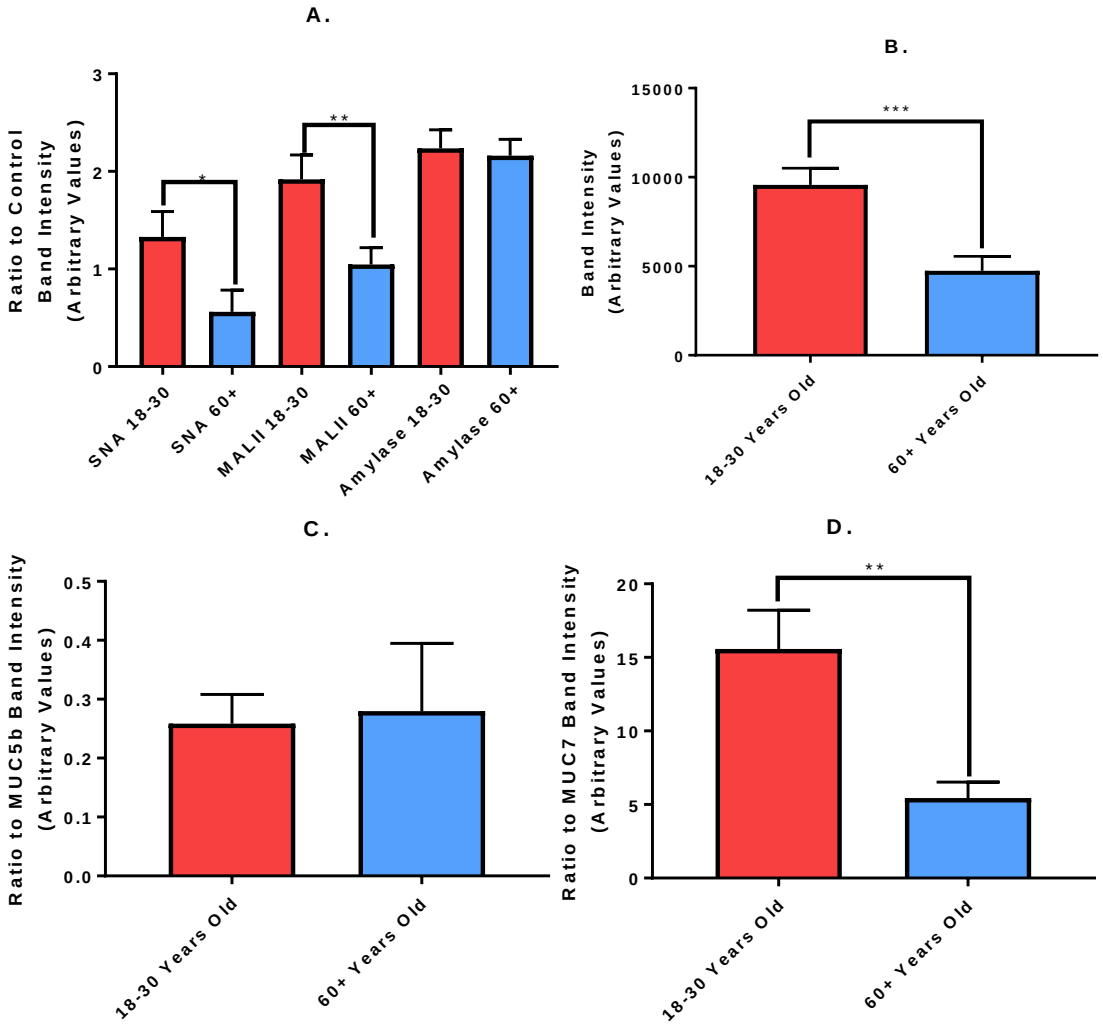


Figure 4-3 MUC5B and MUC in UWMS of older and younger adults determined by immuno-blotting

A. Mean (+/-SEM) MUC5B and MUC7 levels in UWMS from older and younger subjects, determined by immuno-blotting. Band intensity expressed as a ratio to band intensity of control UWMS sample. B. Comparison of mean (+/-SEM) MUC5B and MUC7 levels in UWMS from 18-30 years old group (n=31) determined by immuno-blotting and PAS stained SDS-PAGE gel. C. Ratio of MUC5B and MUC7 levels in UWMS from 18-30 (n=31) and 60+ years old group (n=25) between immuno-blotting and PAS stained SDS-PAGE gel. Difference in means between groups tested for statistical significance using student's T test. P = < 0.05 * P = < 0.01 ** P = < 0.001 *** P=<0.0001 ****. C. Representative image of mucin western blots. MUC5B >250kDa, MUC7 155kDa, Amylase 65kDa (used as loading control), in UWMS from older (60+) and younger (18-30) adults.

4.3.4 Detection of Sialic Acids in UWMS of Older and Younger Adults



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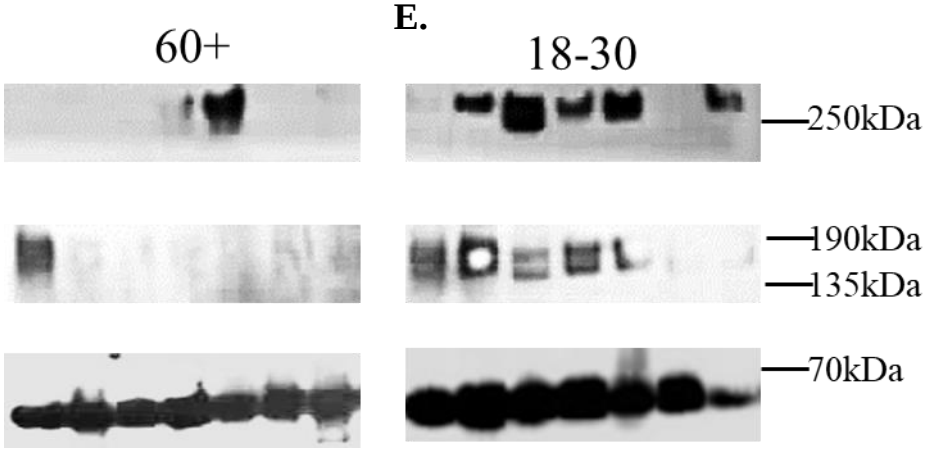

Figure 4-4 SNA and MALII lectin binding in UWMS from older and younger adults

A. Mean (+/-SEM) SNA and MAL II lectin binding in UWMS from older (N=22) and younger subjects (N=31). Mean (+/-SEM) amylase in UWMS, used as loading control. B. Mean (+/-SEM) total sialic acid levels in UWMS from older and younger subjects. Calculated as total band intensity of SNA + total band intensity of MAL II. C. Ratio of SNA lectin to total MUC5B (determined by immuno-blot) in UWMS of older and younger subjects. D. Ratio of MALII lectin to total MUC7 (determined by immuno-blot) in UWMS of older and younger subjects. Difference in means between groups tested for statistical significance using student's T test. P = < 0.05 * P = < 0.01 ** P = < 0.001 ***. E. Representative image of lectin western blots. Detection of sialic acids in UWMS of younger (18-30) and older (60+) adults. SNA >250kDa, MALII 155kDa, Amylase 65kDa.

To further investigate mucin degradation in UWMS from older and younger adults, lectin immuno-blotting was conducted with SNA and MAL II to detect changes in sialylation. There were significantly higher levels of both SNA and MAL II binding of UWMS of younger adults compared to older (Figure 4-4 A). For SNA, the mean (+/- SEM) ratio to the control UWMS was 1.33 +/- 0.22 in younger adults and 0.56 +/- 0.26 in older adults, P=<0.05. For MAL II, the mean (+/-SEM) ratio to the control UWMS was 1.92 +/- 0.17 in younger adults and 1.046 +/- 0.2498 in older adults, P=<0.01. Levels of salivary amylase, used as a loading control, did not differ significantly between groups (Figure 4-4 A). Band intensities of both lectins were combined to give a value for total sialic acid in UWMS from both age groups (Figure 4-4 B). The level of total sialic acid was significantly higher in UWMS of younger adults, with a mean (+/-) band intensity of 9565 +/- 939.5 compared to 4741 +/- 816.3 in the older group (arbitrary values), P=<0.001. When expressed as a ratio to total mucin levels, the ratio of SNA binding to MUC5B did not differ between groups (Figure 4-4 C). The ratio of MAL II binding to MUC7 was significantly higher in the younger group, 15.57 +/- 1.08 compared to 5.45 +/- 2.64 in the older group, P=<0.01 (Figure 4-4 D).

4.3.5 Correlating Physical Properties of Saliva with Mucin Composition

To determine the link between salivary mucin composition and rheological properties of UWMS, bi-variate correlation analysis was conducted. Table 4-2 shows the correlations between spinnbarkeit of UWMS and total levels of MUC5B/MUC7 binding and sialic acid levels in saliva from adults aged between 18 and 60 years. MUC7, determined by PAS staining and immuno-blotting, was positively correlated with spinnbarkeit, with a correlation coefficient of 0.267 ($p=<0.01$) and 0.233 ($p=<0.05$), respectively. Total protein levels in UWMS were negatively correlated to spinnbarkeit, with a correlation coefficient of -0.203 ($p=<0.05$). Levels of MUC7 linked sialic acid, as determined by MALII binding, were also positively correlated with spinnbarkeit of UWMS with a coefficient of 0.212 ($p=<0.05$), as was total sialic acid which had a coefficient of 0.289 ($p=<0.01$). There was no correlation between MUC5B or MUC5B-linked sialic acid levels (SNA) and spinnbarkeit of UWMS.

For viscosity of UWMS, only MUC5B, measured using immuno-blot, was significantly correlated with a coefficient of 0.191 ($p=<0.05$) (Table 4-3). There was no significant correlation between UWMS viscosity and sialic acid content or MUC7 in UWMS.

Table 4-2 Kendall's tau b correlation between UWMS spinnbarkeit and mucin composition.

Levels of MUC5B and MUC7 in UWMS measured using PAS staining of glycoproteins (PAS) and immuno-blotting using antibodies directed to the peptide cores (blot). SNA and MALII lectin binding were quantified as a measure of MUC5B and MUC7 linked sialic acids, respectively, and the two combined to give a value for total sialic acid (SA). Data from both age groups combined, n=54. *denotes statistical significance, P = < 0.05 * P = < 0.01 ** P = < 0.001 *.**

			Spinnbarkeit resting
Kendall's tau_b	Total Protein Resting Saliva	Correlation Coefficient	-.203*
		Sig. (2-tailed)	.027
		N	56
	MUC5B PAS	Correlation Coefficient	.075
		Sig. (2-tailed)	.416
		N	56
	MUC7 PAS	Correlation Coefficient	.267**
		Sig. (2-tailed)	.004
		N	56
	MUC5B blot	Correlation Coefficient	.115
		Sig. (2-tailed)	.217
		N	55
	MUC7 blot	Correlation Coefficient	.233*
		Sig. (2-tailed)	.012
		N	55
	SNA	Correlation Coefficient	.090
		Sig. (2-tailed)	.326
		N	56
	MALII	Correlation Coefficient	.212*
		Sig. (2-tailed)	.021
		N	56
	Total SA	Correlation Coefficient	.289**
		Sig. (2-tailed)	.002
		N	56

Table 4-3 Kendall's tau b correlation between UWMS viscosity (dynamic viscosity, measured using DDM) and mucin composition.

Levels of MUC5B and MUC7 in UWMS measured using PAS staining of glycoproteins (PAS) and immuno-blotting using antibodies directed to the peptide cores (blot). SNA and MALII lectin binding were quantified as a measure of MUC5B and MUC7 linked sialic acids, respectively, and the two combined to give a value for total sialic acid (SA). Data from both age groups combined, n=54. *denotes statistical significance, P = < 0.05 * P = < 0.01 ** P = < 0.001 *.**

			Viscosity in UWMS
Kendall's tau_b	Total Protein Resting Saliva	Correlation Coefficient	.136
		Sig. (2-tailed)	.146
		N	54
	MUC5B PAS	Correlation Coefficient	.087
		Sig. (2-tailed)	.351
		N	54
	MUC7 PAS	Correlation Coefficient	.055
		Sig. (2-tailed)	.556
		N	54
	MUC5B blot	Correlation Coefficient	.191*
		Sig. (2-tailed)	.042
		N	54
	MUC7 blot	Correlation Coefficient	-.031
		Sig. (2-tailed)	.737
		N	54
	SNA	Correlation Coefficient	.068
		Sig. (2-tailed)	.469
		N	54
	MALII	Correlation Coefficient	-.029
		Sig. (2-tailed)	.760
		N	54
	Total SA	Correlation Coefficient	.013
		Sig. (2-tailed)	.887
		N	54

4.3.6 Assessment of Salivary Mucin Muco-Adhesion to an Oral Epithelium Cell Model

To optimise the *in vitro* model for muco-adhesion of saliva, control UWMS from one healthy donor was used on SCC090 and TR146/MUC1 cells. Cells were incubated with saliva for 2 hours and then washed to remove un-bound saliva. The residual, bound, saliva was determined by immuno-blotting of mucins. Both cell lines exhibited binding to salivary MUC5B, although binding was greater in the TR146/MUC1 cells (Figure 4-5). Bound MUC7 was not detected in either cell lines (Figure 4-5 B). MUC1 expression was seen in both cell lines (Figure 4-5 B). As such, volunteer UWMS samples from both age groups were used for the assay in TR146/MUC1 cells. There was significantly greater binding of MUC5B from UWMS of younger adults compared to older, 1.62 +/-0.30 and 0.5986 +/- 0.10 respectively, P=<0.01 (Figure 4-6).

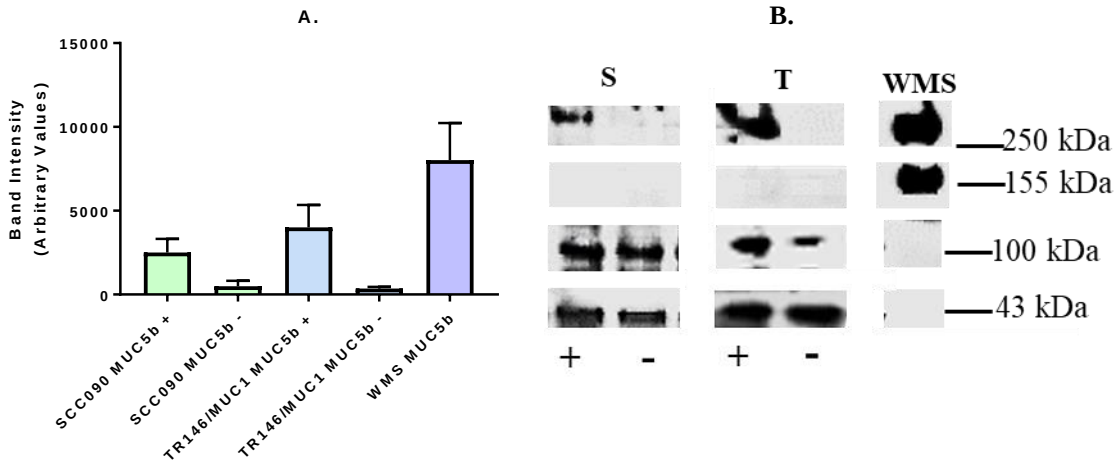

Figure 4-5 Binding of MUC5B from control UWMS to oral epithelial cells
A. Mean (+/-SEM) binding of MUC5B from control UWMS in SCC090 and TR146/MUC1 cells. Control WMS sample used as comparison. B. Immuno-blot images of bound MUC5B and MUC7 from control UWMS to SCC090 (S) and TR146 MUC1 (T) cells. MUC5B=>250kDa, MUC 7 =155kDa, MUC1=120kDa, beta actin (used as loading control)=43kDa. + = with UWMS, - = media only control, WMS = Control WMS sample used as comparison.

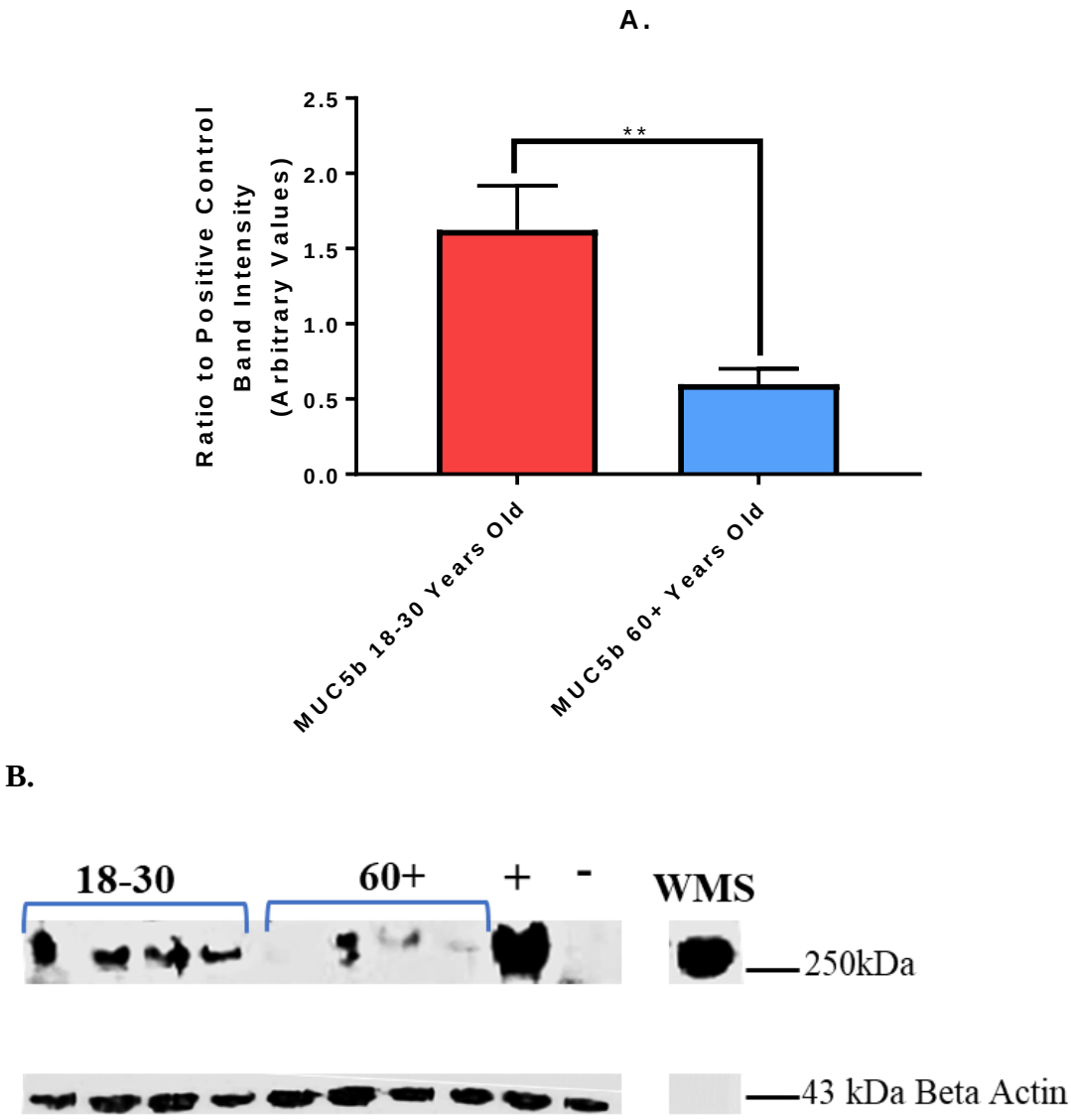

Figure 4-6 Binding of MUC5B from UWMS of older and younger adults to oral epithelial cells

A. Mean (+/-SEM) binding of MUC5B from UWMS of older (N=21) and younger (N=29) adults to TR146/MUC1 cells. B. Representative image of immuno-blot for MUC5B (>250kDa) from UWMS of older and younger adults, binding to TR146/MUC1 cells. + = binding of control UWMS sample, - = negative control (no WMS), WMS = control WMS sample run as a comparison. Beta actin (43kDa) run as loading control. Difference in means between groups tested for statistical significance using student's T test. P = < 0.05 * P = < 0.01 **.

4.4 Discussion

4.4.1 Physical Properties of Saliva in Older and Younger Adults

Viscosity of UWMS samples from older and younger adults was measured using DDM. The viscosity of saliva was around three times greater than the viscosity of water. Other studies, which have used particle tracing to measure mucin rheology have shown that, in purified MUC5B gels, the diffusion coefficient is four times greater than water (Wagner et al. 2017). Measured salivary viscosity values agreed with several previous studies for healthy adults as UWMS from younger adults had a mean viscosity of 1.7mPa.s and from older adults a mean viscosity of 1.6mPa.s was measured. Van der Reijden at al (1993, 1994) reported salivary viscosity to be between 1.5 and 1.8 mPa.s (Van der Reijden et al. 1993, Van der Reijden et al. 1994). Waterman et al (1988) and Nordbö et al (1984) reported average salivary viscosity values of 1.1 and 1.3mPa.s respectively (Waterman et al. 1988, Nordbö et al. 1984). However, others have shown much higher values between 2.5 and 5.7mPa.s (Vissink et al. 1984, Veerman et al. 1989, Rantonen and Meurman 1998). Variation between studies exists because of the lack of standardisation in testing methods and parameters such as temperature and shear rate.

There was no significant difference in UWMS viscosity between age groups which corroborates previous work by Briedis et al who showed no difference in SWMS viscosity between older and younger adults (Briedis et al. 1980). Generally, viscosity of saliva was higher in the younger group however this may reflect the fact that viscoelasticity was also greater in UWMS of younger adults. DDM may be affected by viscoelasticity of the sample since movement of the colloid particles may be different in an elastic fluid compared to a purely viscous sample, known as Brownian motion (Martin et al. 2002). In a purely viscous fluid, the suspended particles lose all recollection of their previous location and are displaced randomly in the fluid (Lai et al. 2009). However, a particle suspended in an elastic fluid is also subjected to an opposing force that pushes the particle promptly back to its initial position (Lai et al. 2009). Therefore, in a solely viscous fluid, displacement increases in a linear manner with time, but displacement is relative to elasticity in a fluid with an elastic component (Lai et al. 2009). However, the effect of viscoelasticity should be minimal in this study as samples were frozen and defrosted before the measurement. Viscoelasticity of saliva is known to be depleted rapidly following expectoration. Following expectoration there is a reduction in the molecular weight of mucin aggregates indicating breakdown of the

supramolecular structure which is responsible for the elastic gel network (Wagner and McKinley 2017). Viscosity is the effect of solutes in a solution while viscoelasticity results from the conformation of salivary mucin (Demouveaux et al. 2017). Therefore, freeze-thawing may alter the conformation of mucin (Francis, Hector and Proctor 2000) so that frozen samples may have little or no visco-elastic properties but retain viscosity as the concentration of solutes would not change (Jaedicke, Taylor and Preshaw 2012, Schipper et al. 2007). Furthermore, the Brownian or α coefficient, which represents anomalous sub-diffusion - the random movement of particles in a fluid with the same density as the particle, can show artefact from elasticity (Martin et al. 2002). In this study the α coefficient was not significantly different between age groups which suggests that the effect of viscoelasticity was similar between groups (Appendix 10). The α coefficient is equal to 2 when particles are freely diffusive, for example in water. Overall the α coefficient was close to 2 in both groups, suggesting there was little effect of viscoelasticity in the viscosity measurements. In line with this, the Spinnbarkeit of UWMS could not be correlated to the viscosity of UWMS (Appendix 10).

DDM is a useful tool for measuring viscosity of low volume samples, demonstrated by the results presented here. It may present a method for measuring bulk rheology of saliva samples from xerostomic patients where the sample volume would otherwise be too small for traditional viscosity techniques. Furthermore, it is a simple technique which is not affected by shear rates and temperature is controlled for in the calculations. As such, it may provide a standardised method for testing viscosity of biological samples which would allow for direct comparisons to be made between studies. However, saliva has a complex surface rheology and high viscoelasticity, therefore further work would be required to see the effect of this on viscosity values obtained from DDM if an absolute viscosity is required.

4.4.2 Salivary Mucin Composition in Older and Younger Adults

Extensional rheology of UWMS could be correlated to levels of MUC7, but not MUC5B, in agreement with previous studies (Inoue et al. 2008). Mucins form a visco-elastic gel like network, with both intra- and inter-molecular disulphide bonds, stabilized by hydrophobic or electrostatic links (Demouveaux et al. 2017). Sialic and sulphate residues, are exposed on the outside of the mucin gel, conferring a net negative surface charge, responsible for electrostatic repulsions which allow for formation of a random coil structure in aqueous medium, such as saliva (Davies et al. 2016). As such,

sialic acids are vital for visco-elastic properties of salivary mucin. Indeed, there was a positive correlation shown in this study, between α 2-3 linked-sialic acid levels and spinnbarkeit of UWMS.

The fact that UWMS from the younger group had significantly more MUC7 than the older group could be anticipated since the ER was also significantly greater in the younger group. Additionally, previous studies have also shown reduced levels of mucin in saliva of older adults, especially MUC7 (Denny et al. 1991, Navazesh et al. 1992).

There were no significant differences in mucin levels in SWMS in either group, however, a trend was apparent since capsaicin rinsing, which evoked a highly viscoelastic saliva in the younger group, also evoked saliva with the greatest levels of MUC7 compared to water rinse and all other rinses. Lack of significant difference in mucin levels in SWMS may be due to high inter-individual variation in salivary composition. This might also be reflective of high inter-individual variation in responses to stimulation as altered composition may be a direct result of SWMS secretion in varying proportions from the different saliva glands.

Generally, there were high levels of inter-individual variation especially amongst the older group for salivary flow rate, protein levels and mucin amounts. This highlights the lack of homogeneity in salivary properties amongst the older population. This high level of inter-individual variation has been highlighted in previous studies and as such, heterogeneity in salivary properties following stimulation could be due to high inter-individual variation in oral sensations in older adults (Boesveldt et al. 2018).

4.4.3 De-glycosylation of Salivary Mucin in Older and Younger Adults

De-glycosylation of salivary MUC5B may have occurred in UWMS from both age groups, since relative levels were lower when determined by PAS staining, which relies on glycosylation, compared to those determined by immuno-blot for the peptide core. Furthermore, MUC7 levels in UWMS were also lower in PAS stained gels compared to MUC7 immuno-blots, in younger adults but not in older. Previous studies have also shown reduced MUC7 levels in UWMS of older adults when measured using stains-all staining for glycoprotein (Denny et al. 1991, Navazesh et al. 1992). Mucin degradation is sequential and begins with de-sialylation, followed by degradation of oligosaccharide side chains which exposes the protein core to proteolytic enzymes (Derrien et al. 2010). UWMS MUC7 may have already been de-glycosylated in the older adults upon

expectoration and further degradation during sample handling would therefore involve proteolysis. Meanwhile, in the younger adults MUC7 may have become de-glycosylated mainly during handling of the samples, although samples were kept on ice during handling to minimise this. This could provide a reason why there were lower levels of MUC7 in older adults compared to younger, assessed through both methods, indicating not only de-glycosylation but also possible proteolysis of the peptide core. Indeed, when levels of glycosylated MUC7 (determined by PAS staining) were expressed as a ratio to total MUC7 (determined by western blot), there was significantly reduced glycosylation of MUC7, in UWMS from older adults. However, western blots and PAS stained gels may not be directly comparable as the two techniques are different and the quantification is not absolute from either method. Therefore, it is also possible that apo-mucin synthesis is reduced in older adults. The proportions of saliva originating from the mucin secreting glands are lower in older adults compared to younger (Affoo et al. 2015). As such, saliva from older adults may contain a greater proportion of parotid saliva, which does not contain mucin, compared to the saliva of younger adults. This may cause reduced levels of MUC7 in saliva from older adults. If this is the case however, then it is not clear why MUC5B was not reduced in saliva of older adults since it is also secreted by the submandibular/sublingual glands. Increased proportions of parotid saliva should, therefore, also lead to lower MUC5B levels which was not observed in this study.

In previous work by Takehara et al (2013), MUC7 was shown to be more susceptible to degradation during sample handling than MUC5B, which forms multimers that are protective against degrading enzyme activity (Takehara et al. 2013). However, assuming quantification from PAS gels and immuno-blots are directly comparable, in this study, MUC5B showed greater de-glycosylation than MUC7. None the less, the relative levels of MUC5B were similar regardless of method used to measure and there was no significant difference in MUC5B between age groups.

The method for normalisation between gels/blots may not have been the most appropriate. A saliva sample from a single donor was loaded onto every gel and all results normalised to this sample. However, the mucin levels in that sample could vary as saliva is not a homogenous fluid. Additionally, both methods for determining mucin levels are subject to variation because of differences in transfer efficiency, exposure times of film to blots and image acquisition. Further, densitometry of immuno-blots or

SDS PAGE gels is only semi quantitative. A more suitable control, such as known concentrations of purified mucins, run on every gel, would have allowed more accurate normalisation between gels and calculation of relative mucin concentration in the samples.

Taken together, the results suggest that reduced MUC7 in UWMS of older adults is due to increased degradation and possibly reduced secretion of saliva from the submandibular/sublingual glands. It could be hypothesised, that increased levels of *Streptococci* bacteria in older adults, shown in other studies, is the cause of observed mucin degradation. *Streptococci* are known producers of sialidase enzymes (Byers, Homer and Beighton 1996, Byers et al. 2000, Beighton and Whiley 1990). As such, greater levels of this bacteria may equate to increased glycolytic and proteolytic activity (Baughan et al. 2000). In line with this observation, it was also shown in chapter 3 that cystatin S levels are lower in UWMS of older adults compared to younger. Salivary cystatins are inhibitors of proteases and as such, greater levels of proteolysis of MUC7 in older adults may be in part linked to reduced cystatin S. As cystatin S is mainly expressed by the submandibular/sublingual glands (Isemura, Saitoh and Sanada 1986), reduced levels in saliva of older adults provides further support for the idea that the proportion submandibular/sublingual saliva is reduced in the WMS of older adults. Previously it was suggested that increased salivary cystatin S could inhibit taste responses because of inhibition of proteolysis leading to a thicker saliva, which acts as a barrier to the taste buds (Dsamou et al. 2012). However, it has been shown here that this is not necessarily the case since younger adults, who had greater salivary cystatin S, displayed more elastic but not more viscous saliva. Furthermore, UWMS viscoelasticity, but not viscosity, could be positively correlated to oral stimulus sensitivity in this study (Appendix 11). Participants were classified as high or low sensitivity depending on whether they had a greater or lower perception of each stimulus than the median. The correlation analysis showed that high sensitivity individuals may have greater UWMS spinnbarkeit than low sensitivity individuals, regardless of age. However, viscosity could not be correlated with stimulus sensitivity. Therefore, viscosity may be less important than viscoelasticity when measuring the quality of saliva for sensitivity to oral stimuli.

To further investigate the effect of ageing on degradation of salivary mucin, lectin immuno-blots were conducted using SNA and MAL II to detect α-2, 3 and α-2, 6 linked

sialic acid residues. Levels of both individual sialic acids were lower in older adults compared to younger. When combined to give a value for total sialic acid in UWMS, there were significantly reduced levels in older adults compared to younger. However, when expressed as a ratio of total mucin, to show relative levels, only sialic acid forms recognised by MAL II were significantly different between groups, with a lower ratio in the older adults. This further confirms that MUC7 is degraded in saliva of older adults, since MUC7 sialylation was still reduced even when accounting for the reduced total levels. Therefore, ageing could be linked with reduction in UWMS MUC7 linked sialic acid, reduced PAS staining and levels of peptide core. Reduced sialylation of MUC7 has also been shown in Sjögren's syndrome patients and could affect lubrication and protection of the oral mucosa (Chaudhury et al. 2016). Sialylation of salivary mucin is important for visco-elastic properties of saliva, since degradation of sialic acid residues leads to reduced viscoelasticity (Ito et al. 1988). The negative charge of sialic acids allow for interaction with water molecules, vital for hydration of the mucin gel (Coles et al. 2010).

The impact of degradation of salivary MUC7 in older adults may include reduced functionality and decreased film forming properties of saliva in this age group. It was shown in chapter 3 that viscoelasticity of UWMS was reduced in older adults and this may be because of degradation of MUC7 since this mucin is directly linked to elastic properties of saliva (Inoue et al. 2008). Meanwhile, viscosity of saliva was not altered by ageing. Degradation of mucin would affect conformation of the mucin gel network which is required for viscoelasticity, however concentration of solutes in saliva would not be reduced by this process so viscosity is un-affected (Wagner and McKinley 2017). Thus, older adults may have a less elastic saliva. This could affect taste since viscoelasticity allows for a thin layer of saliva to coat oral surfaces (Veeregowda et al. 2012), required for solubilisation of tastants and transport to the taste buds (Bradley et al. 2003, Matsuo 2000).

Furthermore, mucins are required in saliva to protect oral surfaces from bacterial invasion and damage to the epithelium (Prakobphol et al. 1999, Everest-Dass et al. 2012, Tabak et al. 1982). In older adults this protective layer may be impaired because of degradation of MUC7, and thus, surfaces including the tongue may be susceptible to injury. Specifically, de-sialylation of MUC7 has previously been shown to reduce its bacteria aggregating capacity (Levine et al. 1978, Bucki et al. 2008). Pathogenic

members of the Candida species bind to mucin oligosaccharide structures and are cleared from the oral cavity in saliva (Everest-Dass et al. 2012). De-glycosylation and reduced sialylation of MUC7 in saliva of older adults may therefore contribute to candidiasis in this age group. Indeed, presence of *Candida albicans* on the tongue has been shown to cause reduced taste function with increased thresholds for sour, bitter, sweet and salt tastes (Sakashita et al. 2004). The fact that oral candidiasis has been shown to be more prevalent in adults over 60 years old, adds weight to the idea that MUC7 degradation may impair microbial defences in older adults (Katoh et al. 1997).

Mucin in saliva samples from both age groups may have been subject to degradation during sample handling and storage which could affect the results. In addition to storing saliva samples at -80°C and keeping them on ice when in use, addition of sialidase inhibitors may have further protected against degradation occurring during processing of the saliva.

4.4.4 Muco-Adhesion of UWMS Mucin to the Oral Epithelium

Since muco-adhesion is an important functional property of salivary mucin which may affect taste function, a muco-adhesion assay was performed to assess the effect of altered mucin sialylation and glycosylation on adhesive capabilities of saliva in older adults. TR146 cells which stably express MUC1 were selected as a model for the oral mucosa. Preliminary experiments showed that TR146 cells are capable of binding some salivary mucin and overexpression of MUC1 enhances this function (Appendix 12). Previous studies have also shown that surface bound MUC1 is important for muco-adhesion of mucin since it facilitates mucin-mucin binding to MUC5B (Ployon et al. 2016). In the present study, adherence of UWMS MUC5B from older adults was reduced compared to younger adults. No binding of MUC7 was shown in initial studies using a single donor control saliva sample, therefore MUC7 adherence was not investigated further. These results suggest that despite total levels of MUC5B being similar between age groups, the functionality of this mucin and its capability to bind to the oral epithelium may be impaired in older adults. This may be due to de-sialylation of MUC5B in saliva of the older group since levels of α-2, 6 linked sialic acid (recognised by SNA) in UWMS were lower compared to the younger group. Although, relative to the total amount of MUC5B, α-2, 6 linked sialic acid was not significantly different between groups.

The age-related reduction in salivary muco-adhesion seen in this study could be an underestimate of that occurring *in vivo*, since MUC1 expression has also been shown to be reduced in the oral epithelium of older adults (Chang et al. 2011). Therefore, not only is salivary MUC5B binding capacity reduced in older adults but, lower expression of surface bound MUC1 may further reduce muco-adhesion, *in vivo*.

Reduced binding of MUC5B to the oral epithelium could affect taste function since MUC5B may be responsible for binding of the salivary pellicle to the oral mucosa. MUC5B participates in mucin-mucin binding to surface bound MUC1 and it is through this mechanism that the mucosal pellicle is created (Ployon et al. 2016, Gibbins et al. 2015). If the layer of saliva on the tongue is reduced, because it is thin and easily washed away or because it is not bound to the mucosa through MUC1/MUC5B interactions, dissolution of tastants may be impaired. Increased surface binding of saliva may lead to enhanced retention of taste compounds in the oral cavity, which could perpetuate responses (Malkki et al. 1993, Cook et al. 2018). Lipophilic and hydrophobic compounds for example, bitter tastants such as caffeine, and TRP agonists such as capsaicin, bind to mucin in saliva (Larhed et al. 1997, Norris and Sinko 1997, Matthes et al. 1992). Therefore, if saliva is highly muco-adhesive, these tastants may be retained in the mouth for longer and thus remain available to the taste buds for activation of taste/TRP receptors for a longer period. In chapter 3 it was shown that capsaicin induced SWMS flow rate was significantly lower in older adults, indicating reduced responsiveness in this age group. Indeed, older adults generally display reduced responsiveness to lipophilic taste compounds compared to younger adults, (Schiffman et al. 1994a). It could be proposed therefore that the decreased MUC7 and reduced muco-adhesion, shown here in older adults, leads to reduced mucin interaction with taste/TRP compounds and reduced retention in the mouth which overall causes a lower response.

Of note, in this study, caffeine responsiveness was not greater in younger adults compared to older, despite it being a lipophilic compound which likely binds to salivary mucin. This may be because caffeine, which can inhibit IP_3R3, required for type ii taste cell responses, has a negative response profile (Bezprozvanny et al. 1994, Gees et al. 2014). This means that higher concentrations, such as the supra-threshold concentration used in this study, may be less intense to a healthy individual. Therefore, higher concentrations of caffeine are perceived less intensely in younger adults but in older

adults with taste loss, there may be no such concentration-intensity relationship. Thus, caffeine responses observed in this study were not different between age groups.

Muco-adhesion may also be important for retro-nasal olfaction of flavour compounds, including the menthol used in this study. This is because, retro-nasal olfaction during eating occurs when flavour compounds reach the oral cavity after swallowing (Salles et al. 2011). The exhalation of air then transports the compounds to the nasal cavity, where they are sensed by olfactory receptors. In this study there was a greater salivary response to menthol in younger adults which may indicate increased retro-nasal perception due to enhanced oral retention and muco-adhesion of saliva.

To investigate muco-adhesion on the tongue epithelium, which may be more relative to taste function than the buccal mucosa represented by TR146 cells, the SCC090 cell line was used. SCC090 originate from a squamous cell carcinoma of the base of a human tongue. They are frequently used for cancer research, as a model for head and neck cancers, but their usefulness as a model for taste has not yet been demonstrated. Mucin binding capability of SCC090 cells was shown to be lower than that of TR146/MUC1 cells, likely due to over-expression of MUC1 in the latter, enhancing mucin binding. However, there was evidence of endogenous MUC1 expression in the SCC090 cell line and binding of salivary MUC5B was also demonstrated. Therefore, this cell line may provide a valuable model of the tongue epithelium to allow study of the effects of the mucosal pellicle on the tongue. For this reason, the SCC090 cell line was used alongside TR146/MUC1 cells in further experiments, as a cell-based model for taste loss.

4.5 Concluding Remarks

The results from this chapter show that despite reduced viscoelasticity of saliva, the bulk rheology, viscosity, does not change in ageing. It was also demonstrated that there are physiological changes in the conformation of salivary mucins in older adults which may lead to reduced functionality of these salivary proteins. De-sialylation and de-glycosylation of MUC7 in saliva of older adults could affect the way in which this mucin can protect and lubricate the oral cavity. Indeed, degradation of MUC7 likely leads to reduced viscoelasticity as the conformation of the mucin gel network may be altered. Meanwhile the relative concentration of proteins and other solutes would not be affected, when flow rate is accounted for, and therefore viscosity was not different between older and younger adults. Since viscoelasticity may be a marker of the quality and functionality of saliva, this suggests that along with reduced quantity, evidenced by reduced flow rate, the quality of saliva is also affected by ageing. Muco-adhesiveness of saliva has been cited in previous studies to be an important factor in taste function and this function may be impaired in older adults since binding of MUC5B from UWMS to TR146/MUC1 cells was reduced in this age group compared to younger adults. To further investigate the role of saliva in taste function, and the effect of altered physical properties and mucin functionality, an *in vitro* model was created using the TR146 and SCC090 cell lines and saliva from both age groups.

5 Development of a Model of Saliva-Taste Receptor Interactions

5.1 Introduction

In chapters 3 and 4 it was shown that the physical properties of saliva and mucin composition, were altered in older adults and this may affect oral sensation. To further characterise the effect of altered mucin and salivary physical properties on taste function, an *in vitro* model was developed for analysis of the effect of saliva on diffusion of tastants to a taste receptor.

Several previous studies have used cell-based models for taste receptor activation (Brockhoff et al. 2007, Bufe et al. 2002, Tsien et al. 1985, Meyerhof et al. 2010). Such models allow for study of the taste system, at the receptor level. The human embryonic kidney cell line, HEK293T, has been widely used for this since the cells are readily transfected and are easy to grow (Thomas and Smart 2005). Cells have been transiently transfected with human taste receptor plasmid DNA and receptor membrane targeting studied by altering amino acids using amino-terminal region of the rat somatostatin receptor 3 (Ammon et al. 2002, Brockhoff et al. 2007). A heterologous expression system is created using co-transfection with a G protein, usually gustducin, since taste responses have shown to be reduced by 70% in gustducin knockout mice (Caicedo et al. 2003). Furthermore, introducing a single mutation in the C-terminus of the alpha gustducin gene leads to reduced sweet and bitter taste responses in mice (Ruiz-Avila et al. 2001).

Generally, responses of transfected receptors to taste ligands are measured by quantifying intracellular calcium concentrations, as the main secondary messenger. Transfected cells are stained using a calcium fluorophore, usually an acetoxymethyl (AM) ester, which is cleaved by intracellular esterases to retain the fluorescent indicator molecules within cells and allow dynamic interactions with calcium ions (Gee et al. 2000). Various methods for measuring changes in intracellular calcium exist, including fluorescent microscopy (Chandrashekar et al. 2000), fluorescence imaging plate readers (FLIPR) (Brockhoff et al. 2007, Bufe et al. 2002) and the FLEX station micro-plate reading system (Narukawa et al. 2011, Upadhyaya et al. 2015).

However, use of a kidney cell line may not be a good representation of the oral cavity or indeed, the tongue and thus may not be representative of oral taste receptor activation *in vivo*. Furthermore, little consideration is given to the effect of the salivary pellicle on transduction of tastants to the receptor. Saliva plays an important role in solubilisation and diffusion of tastants to the taste buds *in vivo* (Matsuo 2000). As such, when creating an *in vitro* model of taste, which closely mimics the environment of the oral cavity, it is important to consider the effect of the salivary mucosal pellicle.

Previous studies have demonstrated that bitter taste may be least well retained in older adults (Yoshinaka et al. 2016). Bitter taste allows for detection of toxic substances and is also involved in acceptance of certain foods such as coffee, wine and vegetables. It is thought that bitter taste responses may facilitate rejection of poisonous substances (Glendinning 1994). Because of the importance of bitter taste in nutrition, and since bitter receptors are well characterised in the literature, the focus of the *in vitro* taste model experiments was on bitter receptor activation. The structures of bitter compounds are diverse and include fatty acids, proteins and amino acids, amines, amides, azacycloalkanes, N-heterocyclic compounds, ureas, thioureas, carbamides, esters, lactones, carbonyls, phenols, crown ethers, terpenoids, secoiridoids, alkaloids, glycosides, anti-oxidants, steroids, halogenated/acetylated sugars, and metallic compounds (Belitz and Wieser 1985).

The detection of bitter tastants occurs via a sub-set of type ii taste receptor cells which express TASTE 2 receptors (TAS2R) (Chandrashekar et al. 2000, Behrens and Meyerhof 2006). TAS2Rs are a family of G protein coupled receptors (GPCRs) of which there are around 25 different receptor types in the human genome (Roper 2007, Meyerhof et al. 2010). GPCRs have 7 transmembrane (TM) spanning domains. Upon binding of the bitter ligand to the binding site on the TAS2R receptor, interaction with heterotrimeric G proteins, namely, Gα-gustducin, Gβ3 or Gβ1, and Gγ13, occurs (Figure 5-1). This leads to dissociation of the βγ subunits of the G protein, which activate phospholipase Cβ2 (Huang et al. 1999). In response, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP^3) are produced from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) (Zhang et al. 2003). Type iii IP^3 receptors (IP_3R3), expressed in the membrane of the endoplasmic reticulum (ER) are activated by IP^3 and intracellular calcium release, from the ER stores, occurs (Clapp et al. 2001). The increase in intracellular calcium leads to activation of transient receptor potential melastatin 4/5

(TRPM4/5) channels which open and allow Na+ influx, causing cell depolarization (Banik et al. 2018). Following this, action potentials are fired, and neurotransmitter release occurs (Huang and Roper 2010). Thus, in this study, intracellular calcium was measured as an indicator of TAS2R receptor activation.

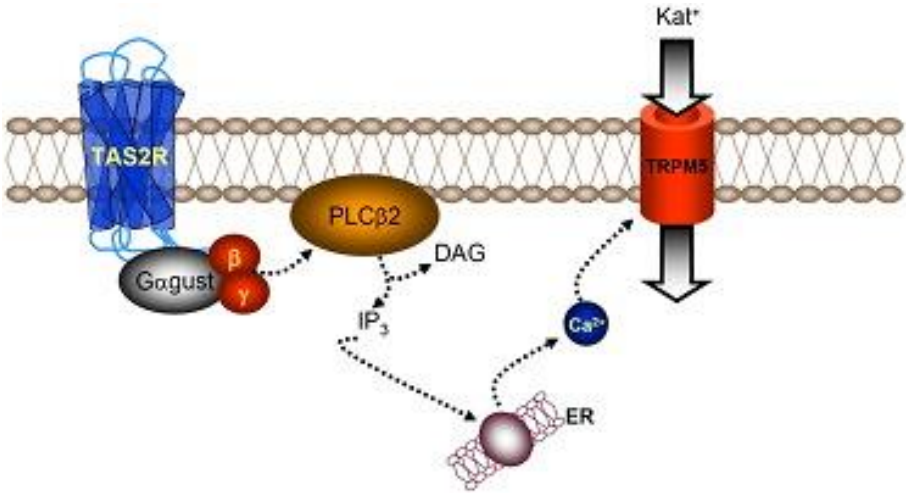

Figure 5-1 (Behrens and Meyerhof 2011) Schematic diagram of the activation of the TAS2R taste receptor pathway

TAS2R38 is a bitter receptor expressed by type ii taste cells and responsible for sensation of bitter compounds containing a thiouracil ring such as PTC and PROP (Bufe et al. 2005, Kim and Drayna 2005). This receptor has been studied in detail because of a common TAS2R38 polymorphism which results in some individuals who cannot taste PTC or PROP, so called non-tasters. Interestingly, in chapter 3, it was shown that PTC sensation was significantly reduced in older adults compared to younger. Therefore, this receptor was chosen to be transfected into oral epithelial cells, to investigate the effects of age-related changes in saliva on TAS2R38 receptor activation. This would allow for understanding of the mechanisms behind age-related changes in responses to TAS2R38 ligands, and how this may be linked to age-related changes in salivary physical properties.

Over-expressing taste receptors in mammalian cells provides a method for study of receptor activation *in vitro*. It allows for non-specific or receptor independent effects of taste compounds to be eliminated as receptor activation in transfected cells can be compared with that in un-transfected or mock transfected cells. Effects in the latter cells are likely to be independent of the taste receptor. Certain taste compounds, particularly small molecule, hydrophobic compounds such as some bitter tastants, are biologically

active and capable of activating various ion channels as well as diffusing through the cell membrane to activate G-proteins directly (Meyerhof et al. 2010, Naim et al. 1994). As such, there may be intracellular calcium responses to those compounds in cellular assays even when expression of the taste receptor does not occur. One such example of this in the literature is the existence of a TAS2R38 independent mechanism for the intracellular Ca^{2+} stimulating effects of PTC. PTC is capable of inducing calcium responses in NCI-H716 cells however, this cell line expresses the non-taster variant of the TAS2R38 receptor, with alanine, valine, and isoleucin at receptor positions 49, 262, and 296, respectively (Dotson et al. 2008, Rozengurt et al. 2006). Furthermore, in human studies, individuals homozygous for the non-taster variant of TAS2R38 display residual sensitivity to both PROP and PTC (Bufe et al. 2005, Kim et al. 2003). Meanwhile, in transfected HEK293T cells, there is a complete absence of calcium responses to PROP or PTC in the non-taster TAS2R38 (Bufe et al. 2005). Therefore, the nature of TAS2R38 independent PTC/PROP responses remains to be elucidated. In STC-1 cells, responses to TAS2R38 agonists could be inhibited by blocking of L-type voltage-sensitive calcium channels, indicating a role for such ion channels in TAS2R independent bitter responses (Chen et al. 2006). TAS2R4, activated by quinine and denatonium benzoate along with several other naturally occurring bitter compounds (Meyerhof et al., 2010), has also been shown to respond to propylthiouracil (PROP) and may therefore be involved in TAS2R38 independent responses to this compound (Chandrashekar et al., 2000).

In addition, bitter taste receptors are found in extra-oral locations. In respiratory cells bitter receptors may function as bronchodilators to reduce airway obstruction (Deshpande et al. 2010). Further, expression of bitter receptors has also been shown in cells of the gastrointestinal tract, where they may play a role in glucose homeostasis (Wu et al. 2002, Rozengurt et al. 2006). One study also showed expression of TAS2Rs in the skin epithelium, where they are suggested to play a role in keratinocyte differentiation (Wölfle et al. 2015). In the work described in this chapter, expression of TAS2R38 was investigated by RNA microarray analysis.

Furthermore, ligand induced stabilisation has been characterised in several other types of GPCR and may lead to increased expression of the receptor in cells which have only a low level of expression (Zhang, Stevens and Xu 2015). Initially the ligand binds to GPCR binding site, which may induce a change in the conformation via protein folding

which locks the receptor in its stable form (Samama et al. 1997). Stabilisation of the receptor conformation can promote membrane surface expression for GPCRs which have fundamentally high basal activity (Gendron et al. 2006, Cahill, Holdridge and Morinville 2007). GPCR ligands can reduce internalisation of the receptor and enhance membrane trafficking (Zhang et al. 2015). Indeed β-adrenergic receptors, expressed at low levels in cardiac cells, can be stabilised by a range of ligands leading to 50-fold increase in receptor expression (Samama et al. 1997). Conversely, some GPCR ligands may also cause internalisation of the receptor which leads to down-regulation due to degradation in the lysosome, following induction of the endocytic pathway during ligand induced internalisation (Koenig and Edwardson 1997, Gray and Roth 2001, Kallal et al. 1998). Some TAS2Rs may be internalised upon agonist stimulation, leading to rapid de-sensitisation, which is dependent on the agonist used (Bufe et al., 2002; Meyerhof et al., 2005; Robinett et al., 2011). 5-Hydroxytryptamine type 2, A (5-HT2A) receptors expressed in smooth muscle cells are down regulated by prolonged exposure to an agonist (Leysen and Pauwels 1990). In cerebellar granule cells, however, 5-HT2A receptors are up-regulated by agonist treatment, in a calcium influx dependent manner (Chen, Li and Chuang 1995). Thus, there are differences in GPCR receptor regulation in various cell systems, but it is clear that agonist treatment can influence receptor expression. For this reason, it was hypothesised that treatment of TR146 cells with a bitter agonist might induce or decrease expression of the TAS2R receptor which, in this study, was found to be expressed endogenously at low level. Significant responses in un-transfected cells, where expression of the receptor is apparently low, would limit the application of this over-expression model. Therefore, a preliminary microarray assessment was performed before and after treatment using the TAS2R38 agonist, PTC, to observe changes in receptor expression upon stimulation with a ligand. These analyses also provided information about potential TAS2R independent response mechanisms to agonists in un-transfected cells.

TR146 cells were shown, in chapter 4, to be a model for the oral epithelium since they facilitate binding of the salivary pellicle. MUC1 over expression enhanced this function, and may provide a model which is closer to the *in vivo* environment, as oral epithelial cells express MUC1 *in vivo* (Hanisch and Müller 2000, Chang et al. 2011). In chapter 4 and in previous studies, MUC1 expression in TR146 cells was shown to be minimal (Ployon et al. 2016). Therefore, although both TR146 variants were characterised, the MUC1 over-expression cell line was selected for development of the model of taste

stimulation, as these cells would facilitate creation of a salivary mucosal pellicle. This would therefore permit the use of saliva samples collected in chapter 3 of this study, to determine the effects of saliva on taste.

Several cell types, which endogenously express TAS2Rs, have been used previously as models of TAS2R receptor activation. These include – the human keratinocyte cell line HaCat, STC-1 cells originating from intestinal tumour tissue of transgenic mice, HuTu-80 cells originating from human duodenal cancer, AR42J cell line originating from rat pancreatic acinar tumours and JEG3 human placental cell line (Wölfle et al. 2015, Wölfle et al. 2016, Chen et al. 2006, Rozengurt et al. 2006, Wu, Chen and Rozengurt 2005). Interestingly, cell lines of different origins have been shown to have different allelic expression of the TAS2R38 receptor. JEG-3 cells express the taster variant (PAV) while HaCaT cells express the heterozygous (PAV/AVI) variant and the human neuronal cell line SK-N-SH express the homozygous non-taster variant (AVI) (Wölfle et al. 2016).

Since human taste tissue is not easily accessible for study of taste receptors, cell lines expressing receptors are a useful tool for the study of the human taste system. However, all cell types previously characterised originate from extra-oral locations. SCC090 cells originate from squamous cell carcinoma at the base of the human tongue. Therefore, it was hypothesised that they may express some taste receptors constitutively. Use of this cell line allowed for assessment of different taste compounds for which the corresponding receptor plasmid was unavailable for use in transfections. Previously, in chapter 4, it was shown that SCC090 cells express MUC1 and facilitate MUC5B binding similarly to the TR146 cell line. Therefore, this cell line was also selected as a model for age-related taste loss. These cells could then be used to create a salivary pellicle, using saliva from older and younger adults, to measure responses to taste compounds for which endogenous expression of the corresponding receptor could be shown.

The aims of this chapter were: i. to create an *in vitro* model for age-related taste loss, which could be used to show the effect of age-related changes in saliva on taste receptor activation -; ii. to characterise taste receptor expression in both TR146/MUC1 and SCC090 cell lines and identify endogenous taste receptor expression.

Micro-array analysis of TR146 cells was conducted - to show total gene expression. Expression of taste receptors was characterised at the protein level using western blotting, and at the mRNA level using RT-PCR. The cell lines were transfected using the TAS2R38 taste receptor and the efficiency of transfection was optimised. Endogenous expression was demonstrated in SCC090 cells, which were used directly as a model for taste. The cell lines were tested, using confocal microscopy and the FLEX station fluorescent plate reader, for intracellular Ca^{2+} responses to individual taste compounds. This demonstrated functional receptor expression in the two cell lines and established the model for use with saliva samples, to demonstrate the effect of saliva on tastant-receptor interaction *in vitro*.

5.2 Methods and Materials

5.2.1 Cell Culture

TR146, TR146 MUC1 and SCC090 cell lines used in experiments conducted for this chapter were cultured as described in chapter 2.

5.2.2 Polymerase Chain Reaction (PCR)

Polymerase Chain Reaction (PCR) was conducted to determine mRNA gene expression in the cells. Details of the method used can be found in chapter 2.

5.2.3 Restriction Digests of DNA

Restriction enzymes were used to confirm presence of the desired plasmid DNA. Plasmid DNA was subjected to restriction digest, as described in chapter 2 and analysed using agarose gel electrophoresis.

5.2.4 DNA agarose gel electrophoresis

DNA was analysed using agarose gel electrophoresis described in chapter 2.

5.2.5 Sequencing of DNA

DNA sequencing analysis was conducted to confirm purification of the plasmid DNA of interest. The technique used is described in chapter 2. Sequencing was performed by Eurofins MWG Operon (Ebersberg, Germany).

5.2.6 Transfection of TR146 and TR146/MUC1 cells

To allow for expression of TAS2R38/Gα16Gust44, TR146 and TR146/MUC1 cells were transfected using plasmid DNA prepared as described above. LipoJet transfection reagent (Promega, Southampton, UK) was used as described in chapter 2. A mock transfection, using an empty vector, was conducted alongside transfection using DNA of interest as a negative control. Empty pcDNA 3.1+ vector (Invitrogen, Carlsbad, CA, USA) was purified and transfected into cells using identical conditions to the TAS2R38/Gα16Gust44. Transfection conditions were optimised by adjusting the LipoJet:DNA ratio and DNA amount.

Table 5-1 Optimisation of transfection conditions used for functional expression assay in TR146 MUC1 cells

Condition	DNA TAS2R38:GA16Gust44 (ng)	LipoJet (ul)
1	100:50	0.1
2	100:50	0.2
3	100:50	0.3
4	200:50	0.1
5	200:50	0.2
6	200:50	0.3
7	100:100	0.1
8	100:100	0.2
9	100:100	0.3
10	200:100	0.1
11	200:100	0.2
12	200:100	0.3

5.2.7 Immuno-Blotting

Cells were grown to confluency, as described above, in 6-well tissue culture plates. Media was removed, and cells were lysed using a modified RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Sigma-Aldrich, Dorset, UK) for 2 minutes on ice before scraping using a cell scraper. Lysates were transferred to Eppendorf tubes and incubated for 20 minutes on ice. The lysates were centrifuged at 13,300G for 10 minutes at 4°C to eliminate cell debris. The supernatant was collected and used for western blotting of taste receptor proteins. Lysates were stored at -20°C between analysis and kept on ice during experimental work to reduce degradation by proteases.

The Bicinchoninic acid assay (BCA assay) was used to determine the total protein concentration of cell lysates. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate protein in cell lysates. A recombinant TAS2R38 protein (Novus Bio, Cambridge, UK), 0.1µg, was used as a positive control for TAS2R38 antibody detection. Western blotting was used to determine expression of TAS2R38 and FLAG tag in cell lysates. Membranes were blocked in 5% (w/v) skimmed milk powder (Marvell, Premier Foods, London, UK) TBS-T solution for 1 hour. Membranes were incubated at room temperature for 1 hour in primary antibodies diluted in blocking buffer. Omission of the primary anti-body was

used as a negative control. Densitometry was conducted to semi-quantify band intensities of immuno-blots. All techniques used were as described in chapter 2.

For specificity testing of the TAS2R38 antibody, a blocking peptide was used, to facilitate pre-absorption and neutralization of the antibody's antigen binding site. The hTAS2R38 primary antibody was generated from rabbits immunized with a KLH conjugated synthetic peptide comprising amino acid residues 304-332 from the C-terminal region of human TAS2R38. A synthetic peptide (unconjugated) comprising residues 305-332 (LifeSpan BioSciences Ltd. Seattle, WA, USA) was used as a competitive inhibitor of antibody binding to TAS2R38 in western blotting. Identical membranes were prepared and incubated with primary antibody for TAS2R38 as described above, but one membrane had 1mg/ml blocking peptide added to the antibody solution. Following incubation with the primary antibody/peptide solution, the protocol for secondary antibody incubation and band detection was as described above.

5.2.8 RNA Extraction

RNA was extracted from cells using the GeneElute Mammalian Total RNA Kit (Sigma, Dorset, UK) following the manufacturer's instructions. The concentration was determined using the NanoDrop and RNA was used for RT-PCR and microarray experiments. Methods used for RNA extraction are described in chapter 2.

5.2.9 Reverse Transcription

cDNA used in PCR and qPCR was synthesised from RNA preparations using reverse transcription, described in chapter 2.

5.2.10 qPCR

For quantitative analysis of gene expression in transfected and un-transfected cell lines, qPCR of cDNA samples was conducted (method described in chapter 2)

5.2.11 Microarray

RNA was extracted from TR146 cells, treated with and without PTC, and sent to the King's College Genomic Centre, Waterloo Campus, for microarray analysis. A full list of expressed genes was provided as well as fold change in gene expression following PTC treatment.

5.2.12 Immuno-cytochemistry

For detection of TAS2R38 protein expression in transfected and un-transfected TR146 cells, immunocytochemistry was conducted using fluorescent confocal microscopy, as described in chapter 2.

5.2.13 Confocal Microscopy

Preliminary experiments were conducted to assess calcium responses to bitter agonists, in TR146 cells. Full details of the method used for confocal can be found in chapter 2. The fluorescence intensity was quantified using NIS elements software (Nikon, UK). The fluorescence in approximately 10 individual cells was analysed and averaged to provide response profiles from each dish.

5.2.14 FLEX Station Intracellular Calcium Measurements

Intracellular calcium measurements were conducted using the FLEX station fluorescence plate reader (Molecular Devices, San Jose, CA, USA) following fura-2 staining of TR146/MUC1 and SCC090 cells. The full method used is described in chapter 2. Briefly, cells were incubated with fura2-am dye solution for 1 hour at 37°C. The dye was removed, and fresh saline solution added. Baseline fluorescence readings (excitation 340/380 nm/emission 520 nm) were taken for 1 second using a FlexStation 3 (Molecular Devices, San Jose, CA, USA). The automatic pipetting system on the FLEX station was set to add PTC/caffeine after 30 seconds of plate reading and readings were taken every 5 seconds for 1-minute following compound addition. Data were analysed using Softmax Pro software (version 7) and expressed as the ratio between 340 and 380 excitation spectra.

5.2.15 Statistics

Microsoft Excel (Version 1804, Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA) was used for data analysis and generation of graphs. The data was tested for normal distribution using the D'Agostino & Shapiro-Wilk normality tests. Data which was not normally distributed was analysed using non-parametric tests, Friedman test with Dunn's multiple comparisons (comparing 2 or more groups) and Mann Whitney U Test (comparing between 2 groups only). Data which was normally distributed was analysed with one-way ANOVA and Dunnett's multiple comparisons test (comparing 2 or more groups) or

independent student’s t test (comparing between 2 groups only). Significance = p value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****.

5.3 Results

5.3.1 Characterisation of Bitter Taste Receptor Expression in TR146/TR146 MUC1 Cells

To characterise suitability of the TR146 cell line as an *in vitro* model of taste receptor responses, expression of TAS2Rs was characterised in TR146/TR146 MUC1 cells. Firstly, constitutive expression was investigated and then cells were transiently transfected with TAS2R38 and Gα16Gust44, as a substitute for gustducin and the efficiency of transfection was analysed by demonstrating expression at mRNA and protein level.

A microarray was performed using RNA extracted from TR146 cells to assess endogenous expression of taste receptors. Expression of 14, out of 25, human TAS2R genes, was shown (Figure 5-2 A). Additionally, the sweet and umami receptors TAS1R1, TAS1R3 and TAS1R2 were expressed, with gene expression values (arbitrary units, $\log_2$ scale) of 2.55, 3.32 and 2.71 respectively. The most prominently expressed TAS2R genes were TAS2R41 and R14 with expression levels of 2.98 and 2.92 (arbitrary values) respectively. For reference, the value for YWHAZ, a common housekeeping gene, was 8.4. The value assigned to the PTC/PROP receptor, TAS2R38, was 2.13. Guanine nucleotide-binding proteins (G proteins) facilitate downstream transduction of G protein-coupled receptors (GPCRs) such as TAS2R receptors. Along with GNB1 (β-subunit) and GNG13 (γ-subunit), G(t) protein α 3 (GNAT3) is a component of the hetero-trimeric G protein, gustducin which frequently associates with TAS2R. Expression of GNB1 and GNAT3 was shown in TR146 cells as well as expression of other G alpha, beta and gamma proteins which may be capable of facilitating taste receptor transduction (Figure 5-2 B). The top 10 % of genes had expression levels greater than 5.8, with a maximum of 12.79.

5.3.2 Analysis of Bitter Taste RNA Expression in TR146 Cells Using Microarray

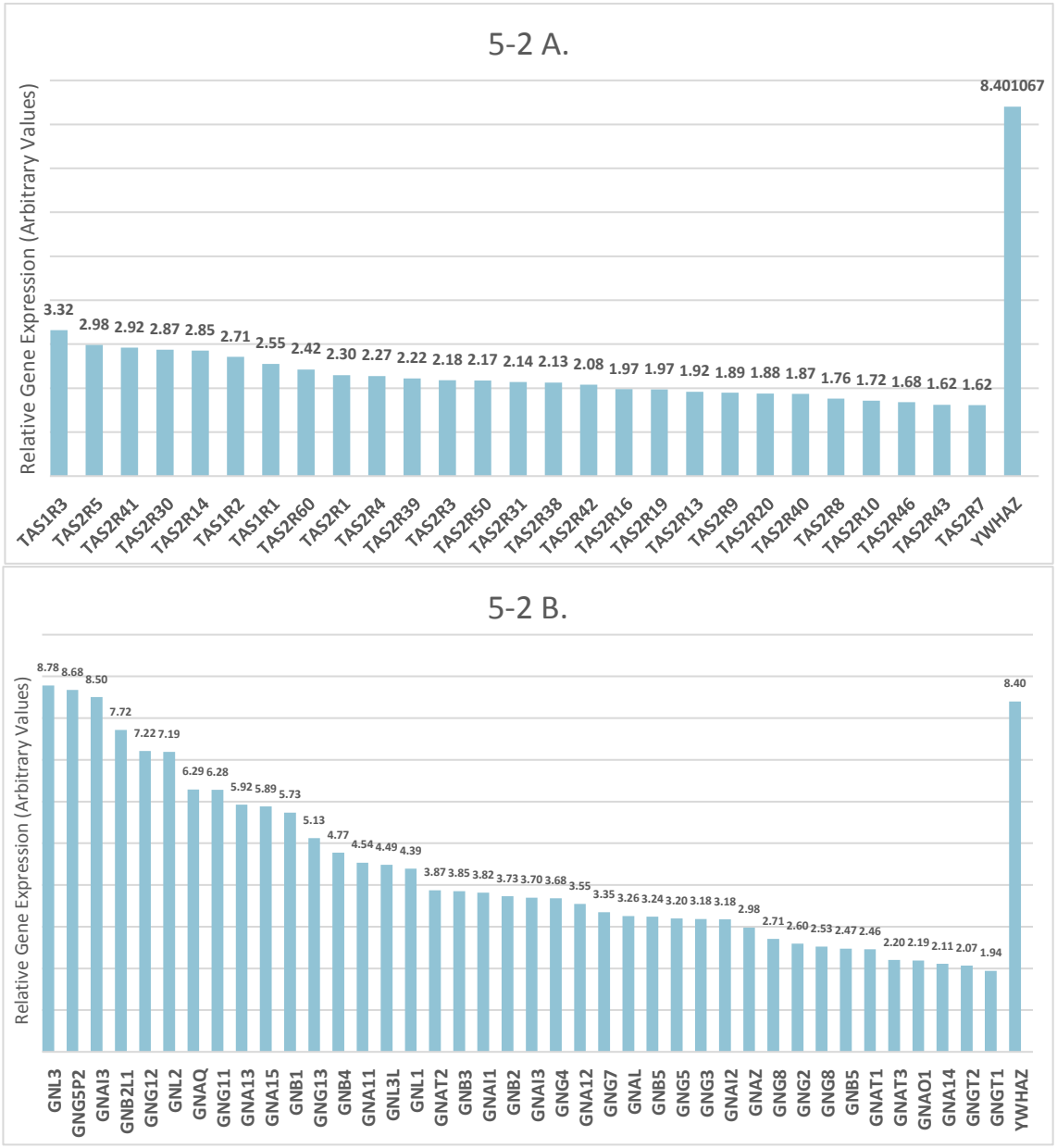

Figure 5-2 Expression of taste receptor genes in human oral epithelial cell line, TR146.

Determined by microarray of TR146 cell RNA. A. Relative levels of TAS2R genes in TR146 cell mRNA. B. Relative levels of GNAT proteins in TR146 cell mRNA.

5.3.3 Investigating changes in mRNA in TR146 Cells Following PTC Stimulation

TAS2R-independent calcium responses to stimulation with bitter agonists, including PTC, have been demonstrated using *in vitro* cell models (Meyerhof et al. 2010). In this study, preliminary calcium response assays found calcium responses to TAS2R38 agonists at concentrations above 100µM in un-transfected TR146 cells (Appendix 13). To determine whether TAS2R38 was expressed in TR146 cells and whether expression of TAS2R receptors or signalling pathways were modulated by exposure to tastants, gene expression was compared in TR146 cells with and without stimulation by the TAS2R38 agonist PTC. Cells were stimulated with 100µM PTC at 2-hour intervals, over an 8-hour period and RNA was extracted for microarray. Gene expression of most genes, approximately 50,000, including TAS2Rs, were not up or down regulated by stimulation with PTC. The expression of several other genes was altered by PTC stimulation. In total, 61 genes were up-regulated following PTC stimulation and 109 genes were down-regulated, with a fold change of 2 or more (Table 9-19, Appendix 14). Out of those genes, 5 up-regulated and 5-down regulated genes could be linked to taste transduction in a literature search (Table 5-2). It is possible therefore that one or a combination of these genes are involved in sensing of PTC/PROP in a TAS2R38 independent pathway.

Table 5-2 Analysis of fold change in genes relevant to chemo-sensation, in mRNA from TR146 cells following stimulation with PTC.

Determined by micro-array of TR146 cell RNA before and after treatment with 100µM PTC.

Fold Change	Gene	Description	Function
3.04	CLK2P1	CDC like kinase 2, pseudogene 1	GPCR kinase, mediate phosphorylation of GPCR's, Amphipathic tastants ↑β2AR signalling and ↓intracellular GPCR kinases = delayed desensitisation (Malach et al. 2015)
2.64	SLC25A6	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6	Membrane transport proteins (Hediger et al. 2004). Na+/H+ exchanger (NHE) activity, maintain cytosolic pH (pHc) and systemic Na+ (Beloto-Silva, Machado and Oliveira-Souza 2011). pHc = blocking potassium/calcium channels →membrane depolarization (Schulz and Münzel 2011). NHE regulates Ca^{2+} entry, activates Na+/Ca^{2+} exchanger = change of pHc and ↑ insulin secretion in pancreatic â-cells (Moulin et al. 2007). Part of the toxin efflux system (Choi et al. 2012).
2.15	P2RY8	purinergic receptor P2Y, G-protein coupled, 8	ATP = neurotransmitter in taste buds (Finger et al. 2005) via purinergic receptors: P2X ionotropic ligand-gated ion channel receptors (P2X1-P2X7) and P2Y metabotropic ligand-gated ion channel receptors (P2Y1-P2Y8) (Ralevic and Burnstock 1998). P2RY8 activation = ↑Ca^{2+} and activation of protein kinase C (PKC) (Fujiwara et al. 2007, Bogdanov et al. 1997). P2Y receptors bind nucleotides = intracellular signalling through phospholipase C, adenylyl cyclase and extracellular signal-regulated kinases (ERKs) (Muscella et al. 2004).
2.14	OR2L3	olfactory receptor, family 2, subfamily L, member 3	Olfactory receptors are 7 transmembrane G-Protein Coupled Receptors recognising odours (Buck and Axel, 1991; Mombaerts, 1999).
2.05	GPR20	G protein-coupled receptor 20	KO of GPR20 = ↑ cAMP production (Hase et al. 2008). Tastants evoke cAMP signal in taste buds (Trubey et al. 2006). See below (melatonin receptor 1B), hetero-dimerisation of orphan GPCRs with non-orphan GPCR's = altered ligand binding.
-2.09	AKR7A2	aldo-keto reductase family 7, member A2	AKR7A2 enzymes have high affinity for succinic semialdehyde (SSA) (metabolite of GABA) (Malaspina et al. 2009). Transformation/detoxification of aldehydes and ketones from metabolism or as nutrient, food, drug, or toxin (Barski, Tipparaju and Bhatnagar 2008).
-2.09	OR7C1	olfactory receptor, family 7, subfamily C, member 1	Olfactory receptors are 7 transmembrane G-Protein Coupled Receptors recognising odours (Buck and Axel 1991, Mombaerts 1999).
-2.25	MTNR1B	melatonin receptor 1B	MT1 receptor stimulation ↑IP^3 levels (Roka et al. 1999). Forms heterodimers with GPR50 (Levoye et al. 2006). GPR50 KO = ↑MT binding for agonist I-MLT. Orphan GPR's (such as GPR20) dimerise with non-orphan GPRC's = altered ligand binding. Bitter and non-sugar sweet tastants and odorants stimulate MT receptors (Zubare-Samuelov et al. 2003). (Lerner et al. 1988).
-2.29	RUNX1-IT1	RUNX1 intronic transcript 1	Causal for affecting Marmite preference (Roos et al. 2017).
-3.01	SRGAP2; SRGAP2B	SLIT-ROBO Rho GTPase activating protein 2; SLIT-ROBO Rho GTPase activating protein 2B	Slit/Robo signalling guide direction of cell migration in peripheral/central branches of sensory neurons (Ma and Tessier-Lavigne 2007).

5.3.4 Analysis of TAS2R38 mRNA Expression in TR146 Cells

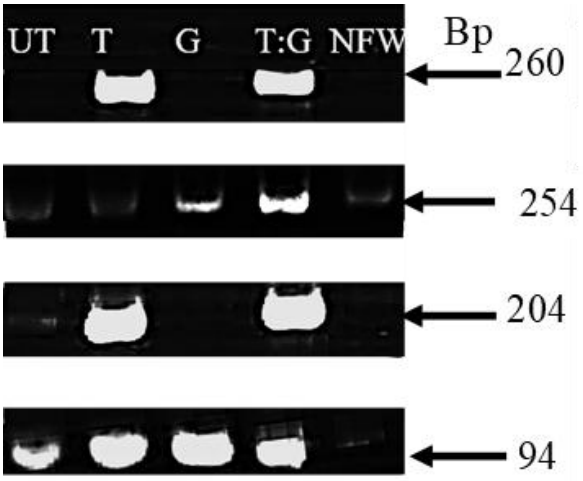

Figure 5-3 RT-PCR amplification of mRNA from TR146 cells, transiently transfected with plasmids encoding hTAS2R38 and the G protein chimera Ga16Gust44.

Representative image of agarose gel of PCR products using TAS2R38 (204Bp), HSV tag (260Bp) and Ga16Gust44 primers (254Bp). YWHAZ housekeeping gene used as cDNA control. UT=un-transfected, T=TAS2R38 transfected, G=Ga16Gust 44 transfected, T/G=TAS2R38:Ga16Gust44 co-transfected, NFW=nuclease free water (negative control). Image represents n = 2-3.

TR146 cells were transiently transfected with plasmids encoding TAS2R38 and Gal16Gust44 and expression was confirmed at the level of mRNA level by RT-PCR. Products of the expected size for TAS2R38 (204 bp) and Ga16Gust44 (254 bp) were amplified using cDNA template from cells transfected singly, with TAS2R38 (lane T) or Ga16Gust44 (lane G). In co-transfected cells both products were amplified (lane T:G) (Figure 5-3). No products were amplified from cDNA derived from non-transfected cells (lane UT).

The TAS2R38 plasmid used for transfection includes the 3’ sequence encoding a herpes simplex virus (HSV) tag. This sequence is not present in chromosomal DNA of TR146 cells. To confirm that amplification products were derived from cDNA, RT-PCR was performed using a 5’primer from the TAS2R38 sequence and a 3’ primer from the HSV sequence. Products of the expected size (260 bp) were amplified from cDNA of transfected but not un-transfected TR146 cells.

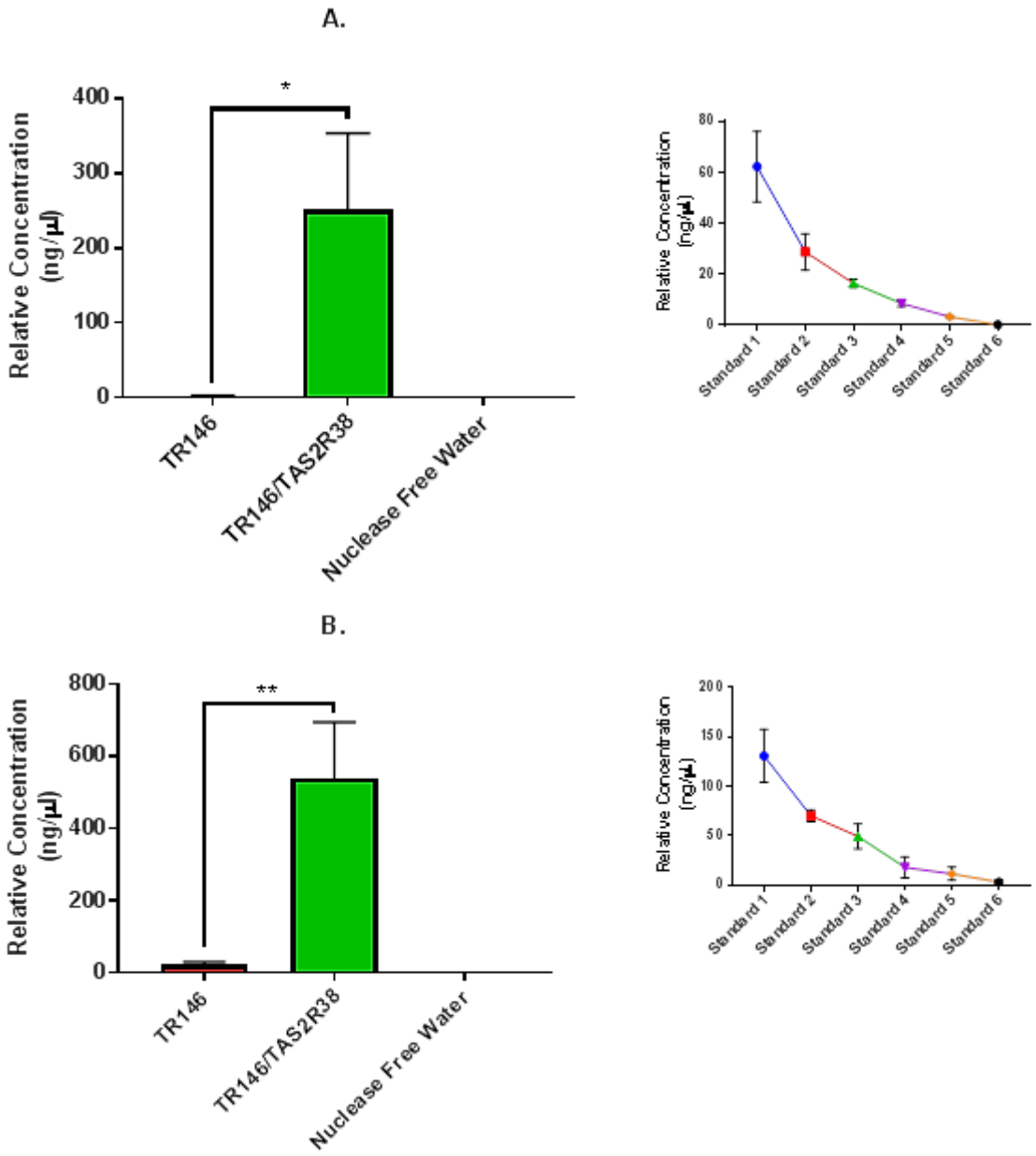

Figure 5-4 qPCR quantification of TAS2R38/HSV gene expression in transiently transfected TR146 cells

Quantification of TAS2R38/HSV gene expression in TR146 cells, transiently transfected with cDNA for TAS2R38 C-terminally extended with an HSV-tag, determined by qPCR. Mean relative concentration (ng/µl) +/-SEM, of TAS2R38 (A.) and HSV tag (B.) cDNA in mock transfected and TAS2R38 transfected TR146 cells. Nuclease free water used as negative control. cDNA from all samples was combined and serially diluted to create a standard curve for TAS2R38 (A.) and HSV tag (B.) gene expression. Relative concentration calculated from known concentration of cDNA (determined by NanoDrop), relative to the expression of YWHAZ housekeeping gene. Statistical significance determined by independent student's T test. Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****. Data represent n = 2-3 (4-6 analyses).

RT-qPCR was also used to further demonstrate and quantify mRNA expression of TAS2R38 and Gα16Gust44 in transiently transfected TR146 cells. Amplification of PCR products was quantified, using a standard curve created by calculating amplification of the gene of interest, normalised to YWHAZ amplification, against the known concentration of total cDNA. Significantly greater mean (+/- SEM) levels of mRNA expression of TAS2R38, 251.7 +/-101.6ng/µl, and HSV tag, 538.7 +/-155.7ng/µl, were shown in TAS2R38 transfected cells compared to mock-transfected (Figure 5-4). Mock-transfected cell mRNA had mean expression values of 2.17 +/-0.89ng/µl for TAS2R38 and 22.8 +/-8.18ng/µl for HSV tag. Additionally, relative expression of Gα16Gust44 was also greater in Gα16Gust44 transfected TR146 cells and TAS2R38:Gα16Gust44 co-transfected cells compared to TAS2R38 and mock transfected (Appendix 15. Figure 9-7), although this was not statistically significant.

5.3.5 Analysis of TAS2R38 mRNA Expression in TR146 MUC1 Cells

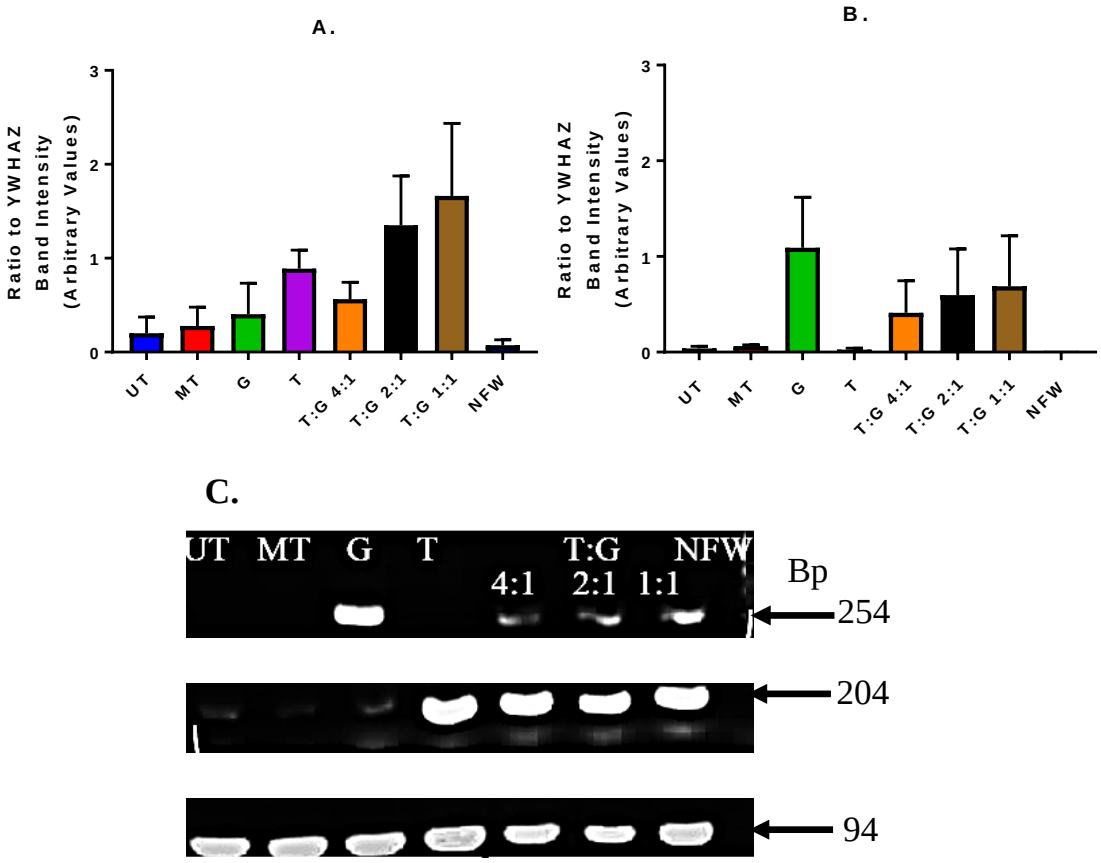

Figure 5-5 RT-PCR amplification of mRNA from TR146 MUC1 cells, transiently transfected with hTAS2R38 and Gα16Gust44.

RT-PCR amplification of mRNA from TR146 MUC1 cells, transiently transfected with cDNA encoding for hTAS2R38 and the G protein chimera Gα16Gust44. Three varying ratios of TAS2R38:Gα16Gust44 were used to optimise co-transfection of cells, 1:1, 2:1 and 4:1. MT=mock transfected, UT=un-transfected, T=TAS2R38 transfected, G=Ga16Gust 44 transfected, T:G=TAS2R38:Gα16Gust44 co-transfected. Semi quantification of band intensity of agarose gel of PCR products using TAS2R38 (204Bp) (A.) and Gα16Gust44 (254Bp) (B.) primers, determined using Image J software. Mean (+/-SEM) band intensity of gene of interest expressed as a ratio to YWHAZ housekeeping gene (94Bp). C. Representative image of agarose gel of PCR products. UT=un-transfected, T=TAS2R38 transfected, G=Gα16Gust 44 transfected, T/G=TAS2R38:Gα16Gust44 co-transfected, NFW=nuclease free water (negative control). Data representative of n = 2-3.

Conditions for optimal transfection of TR146 MUC1 were investigated. Relative mRNA levels were determined semi-quantitatively by measurement of band intensity of RT-PCR amplification products following agarose gel electrophoresis. In cells co-transfected with TAS2R38 and Gα16Gust44 plasmids, levels of Gα16Gust44 mRNA appeared to be lower than in cells transfected with Gα16Gust44 alone (Figure 5-5, panel C, lanes G and T:G 4:1, 2:1, 1:1) however this was not statistically significant (Figure 5-5 panel B, G and T:G 4:1, 2:1, 1:1). Transfection with a 1:1 ratio of TAS2R38 to Gα16Gust44 plasmid DNA resulted in the highest levels of mRNA for both proteins although differences in the ratio were not statistically significant due to the low number of experimental repeats.

Greater expression levels of both TAS2R38 and Gα16Gust44 were shown when a 1:1 ratio of plasmid was used in the transfection (Figure 5-5, panel A and B, lane T:G 1:1). Levels of TAS2R38 and Gα16Gust44 expression were lower in mock transfected cells compared to all other transfection conditions (panel A and B, MT).

5.3.6 Characterisation of TAS2R38 Protein Expression in TR146 Cells

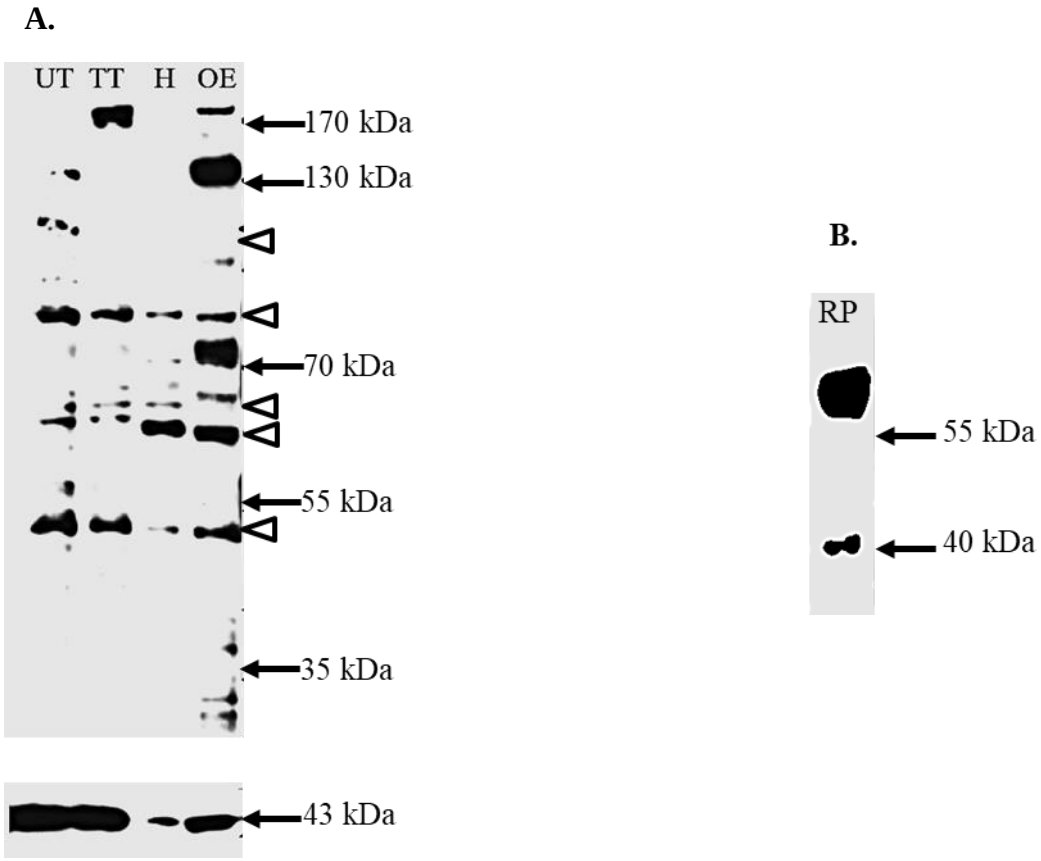

Figure 5-6 Immuno-blotting of whole cell lysates visualized by staining with anti-TAS2R38 antibody.

TR146 cells were transiently transfected with cDNA for TAS2R38 C-terminally extended with an HSV-tag and whole cell lysate used for immuno-blotting. As controls, un-transfected TR146, un-transfected HEK293T whole cell lysate, commercially available TAS2R38 over-expression HEK293T lysate and TAS2R38 recombinant protein are shown in parallel. Predicted molecular weight of receptor oligomers = >100kDa. Predicted molecular weight of full length glycosylated receptor monomers = 44kDa. UT=un-transfected, TT=TAS2R38 transfected, H=HEK293T (negative control), OE=TAS2R38 HEK293T over-expression lysate. B. RP=TAS2R38 recombinant protein used as positive control. 1:500 dilution of primary antibody. Beta Actin used as loading control, expected molecular weight 43kDa. Image representative of n = 2-3.

To create overexpression of TAS2R38 in TR146 cells, transient transfection was performed with TAS2R38 cDNA. This aimed to create an *in vitro* model of taste function as TR146 cells have been used in previous studies as a model for the oral cavity. Protein expression of TAS2R38 was confirmed using western blotting with an antibody raised against a synthetic peptide corresponding to a sequence (residues 305-322) within the c-terminal region of TAS2R38 (Figure 5-6). Specific bands of high molecular weight (approx. 150kDa) were evident in TAS2R38 transfected TR146 cells as well as in a commercially available HEK293T over-expression lysate (lanes TT, OE) likely due to formation of oligomers which are not easily dissociated by heating, in the presence of SDS. Several bands were observed in both negative controls and the expression lysates (indicated by open arrow heads). These presumed non-specific bands could not be eliminated by increased blocking with BSA or milk before probing with antibody nor by increased antibody dilution.

5.3.7 Characterisation of Gα16Gust44 Protein Expression in Transfected TR146 Cells

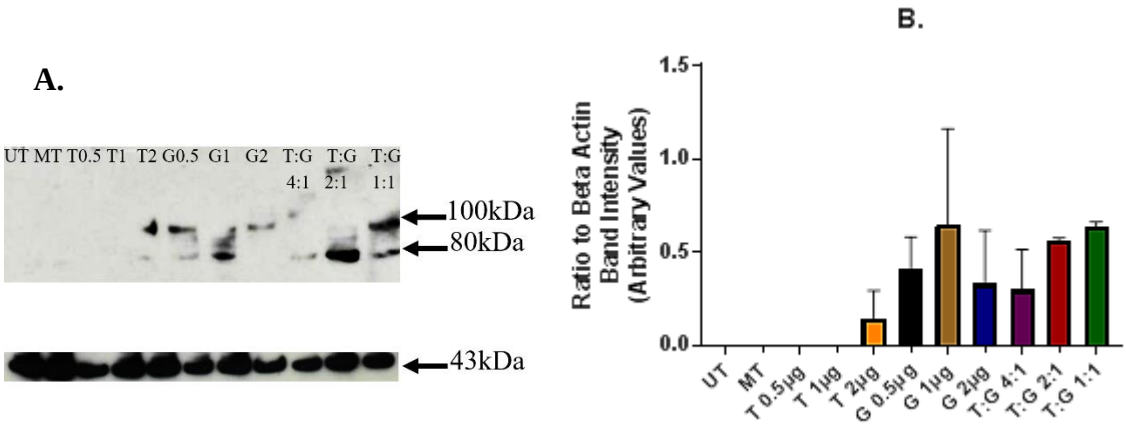

Figure 5-7 Immuno-blotting of whole cell lysates visualized by staining with anti-FLAG tag antibody.

TR146 cells were transiently transfected with three increasing concentrations of TAS2R38 and the chimeric FLAG tagged Gα16Gust44 cDNA and three varying ratios of TAS2R38:Gα16Gust44 cDNA. UT=un-transfected, MT=mock transfected, T=TAS2R38 transfected, G=Gα16Gust44 transfected, T:G=TAS2R38:Gα16Gust44 co-transfected. 1:500 dilution of primary antibody. Representative image of FLAG tag immuno-blot (A.). Mean (+/-SEM) band intensity of FLAG tag expressed as ratio to beta actin loading control (43kDa) (B.). Data represent N = 2.

To show expression of Gα16Gust44 protein in transiently transfected TR146 cells, western blotting was conducted using an antibody directed towards the FLAG tag. The Gα16Gust44 chimera is composed of amino acid residues from the N-terminal region of G16 and the C-terminal sites of gustducin, therefore obtaining an antibody with specificity is challenging. As such, the construct was FLAG tagged to facilitate immuno-blotting detection. Protein bands were seen in lysates from both the Gα16Gust44 and the TAS2R38:Gα16Gust44 transfected cells, indicating successful transfection (Figure 5-7 A). Some cross reactivity may occur between TAS2R38 and the FLAG tag antibody, since cells transfected with TAS2R38 displayed some FLAG antibody staining at the highest concentration of cDNA. There were no significant differences in FLAG protein levels between transfection conditions, however the greatest levels were seen in the 1µg Gα16Gust44 only transfected and the 1:1 TAS2R38:Gα16Gust44 co-transfected TR146 cells (Figure 5-7 B, displayed on representative blot lanes G1 and T:G 1:1).

5.3.8 Optimisation of TAS2R38/Gα16Gust44 Protein Expression in Transfected TR146/TR146 MUC1 Cell Lines

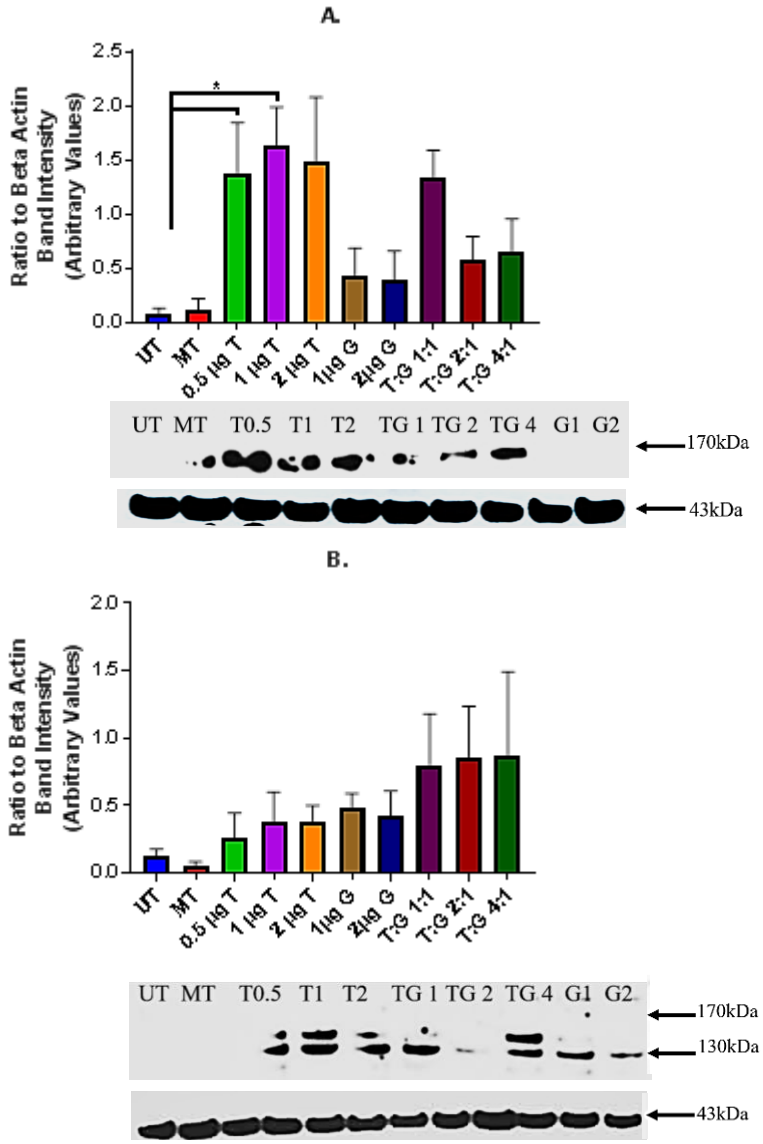

Figure 5-8 Optimisation of transfection efficiency in TR146 and TR146 MUC1 cells.

Increasing concentrations of TAS2R38 and Ga16Gust44 cDNA and varying ratios of TAS2R38:Ga16Gust44 were used to transiently transfect/co-transfect cells. Mean (+/- SEM) band intensity of TAS2R38 determined by immuno-blot in TR146 cells (A.) and TR146 MUC1 cells (B.), expressed as ratio to beta actin loading control (43kDa). Representative image of TAS2R38 immuno-blot also shown for TR146 cells (A.) and TR146 MUC1 cells (B.). Predicted molecular weight of receptor oligomers = >100kDa. Predicted molecular weight of full length glycosylated receptor monomers = 44kDa. UT=un-transfected, MT=mock transfected, T=TAS2R38 transfected, G=Ga16Gust44 transfected, T:G=TAS2R38:Ga16Gust44 co-transfected. P=<0.05*, statistical significance determined using one-way ANOVA with Dunnett's multiple comparisons test. Data represent n = 2-3.

As discussed above, TR146 MUC1 cells may promote formation of a salivary pellicle and therefore provide a better model of the oral epithelium than TR146 cells. This cell line was also used for transfection of TAS2R38 and transfection efficiency was optimised using different concentrations of plasmid. Cells were co-transfected with Gα16Gust44 to create a heterologous expression system, as gustducin enhances TAS2R responses to taste compounds (Ueda et al. 2003). Western blotting, using a TAS2R38 antibody was conducted to semi-quantify protein expression of the TAS2R38 receptor in control and transfected cell lysates. In TR146 cells, mean (+/-SEM) expression of TAS2R38 receptor protein in the TAS2R38 only transfected cells, using 0.5 and 1µg of cDNA, was significantly greater compared to a mock transfection (using an empty vector) or un-transfected cells 1.38 +/-0.46 and 1.64 +/-0.35 compared to 0.12 +/-0.10 and 0.08 +/-0.05 (arbitrary values), respectively (Figure 5-8 A.). Apparently higher levels of expression in the TAS2R38:Gα16Gust44 double transfected cells, compared to mock or un-transfected, were not statistically significant. Expression was highest in the cells transfected with a 1:1 TAS2R38 to Gα16Gust44 ratio, with relative expression of 1.34 +/-0.25. Of note, transfection of cells with Gα16Gust44 only, also increased expression of TAS2R38 slightly compared to mock and un-transfected cells, with an average expression level of 0.41 +/-0.26 in Gα16Gust44 transfected TR146 cells. In TR146 MUC1 cells, greater expression levels of TAS2R38 protein were shown in the double transfection compared to mock or un-transfected cells with an average expression level of 0.85 +/-0.46 in Tas2R38:Gα16Gust44 transfected, compared to 0.12+/-0.06 and 0.05 +/- 0.03 in un-transfected and mock-transfected cells (Figure 5-8 B). Greater levels of TAS2R38 were also seen in the TAS2R38 only and Gα16Gust44 only transfected cells, although to a lesser extent, with respective expression levels of 0.34 +/-0.17 and 0.45 +/-0.15 on average. The differences between transfection conditions were not statistically significant in TR146 MUC1 cells.

5.3.9 Confirmation of Specificity of TAS2R38 Antibody Using Immunising Peptide

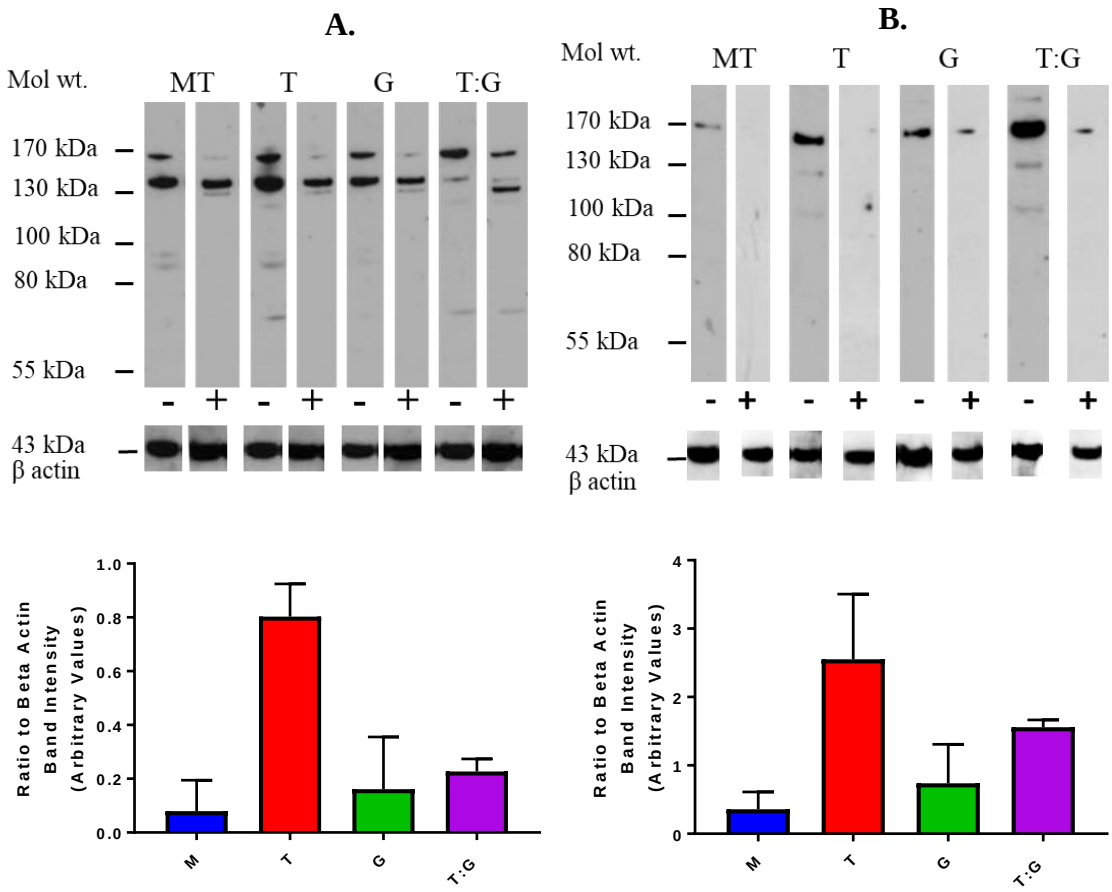

Figure 5-9 Immunising peptide against TAS2R38 was used to determine antibody specificity.

Representative immuno-blot image of TAS2R38 in transfected TR146 (A.) and TR146 MUC1 (B.) cells shown without (-) and with (+) immunising peptide added to the primary antibody to block non-specific binding. Re-probing of the same blots with beta actin was used as a loading control =43kDa. MT=mock transfected, TT=TAS2R38 transfected, G=Ga16Gust44 transfected, TG=TAS2R38/Ga16Gust44 co-transfected, S=SCC090 cells, RP=TAS2R38 recombinant protein. Mean (+/-SEM) band intensity (sum of 2 identified bands) of TAS2R38 in transfected TR146/TR146 MUC1 cells expressed as ratio to beta actin loading control. Intensity values adjusted for non-specific binding by removing the band intensity from immunising peptide blocked blots. MT=mock transfected, T=TAS2R38 transfected, G=Ga16Gust44 transfected, T:G=TAS2R38:Ga16Gust44 co-transfected. Data represent n = 2-3.

Since the TAS2R38 immuno-blot showed multiple bands, the immunising peptide was used to inhibit binding of the TAS2R38 antibody. This also confirmed band positions of TAS2R38 specific protein fragments. There was reduced band intensity of the 2 high molecular weight bands (130 and 170kDa, approximately), following peptide blocking of TAS2R38 (Figure 5-9). When the band intensity for the peptide blocked western blot was subtracted from the non-blocked blot, there was still a greater mean (+/-SEM) band intensity in the TAS2R38 transfected TR146 MUC1 cell lysate compared to mock transfected, 2.56 +/- 0.95 compared to 0.36 +/-0.25 (arbitrary values) (Figure 5-9 B). Greater band intensity was also seen in the Gα16Gust44 co-transfected TR146 MUC1 cells, 1.56 +/- 0.11, and to a lesser extent, in the Gα16Gust44 transfected, 0.74 +/- 0.57 compared to mock transfected. There was also a greater band intensity seen in TAS2R38 only and co-transfected TR146 cells compared to mock transfected, 0.80 +/- 0.12 and 0.23 +/- 0.05 respectively compared to 0.08 +/-0.12. Due to low number of replicates, the results were not statistically significant but go towards demonstrating over-expression in the transfected cells.

5.3.10 Screening for TAS2R38 Receptor Protein Expression in Transfected TR146 Cells by Immunocytochemistry

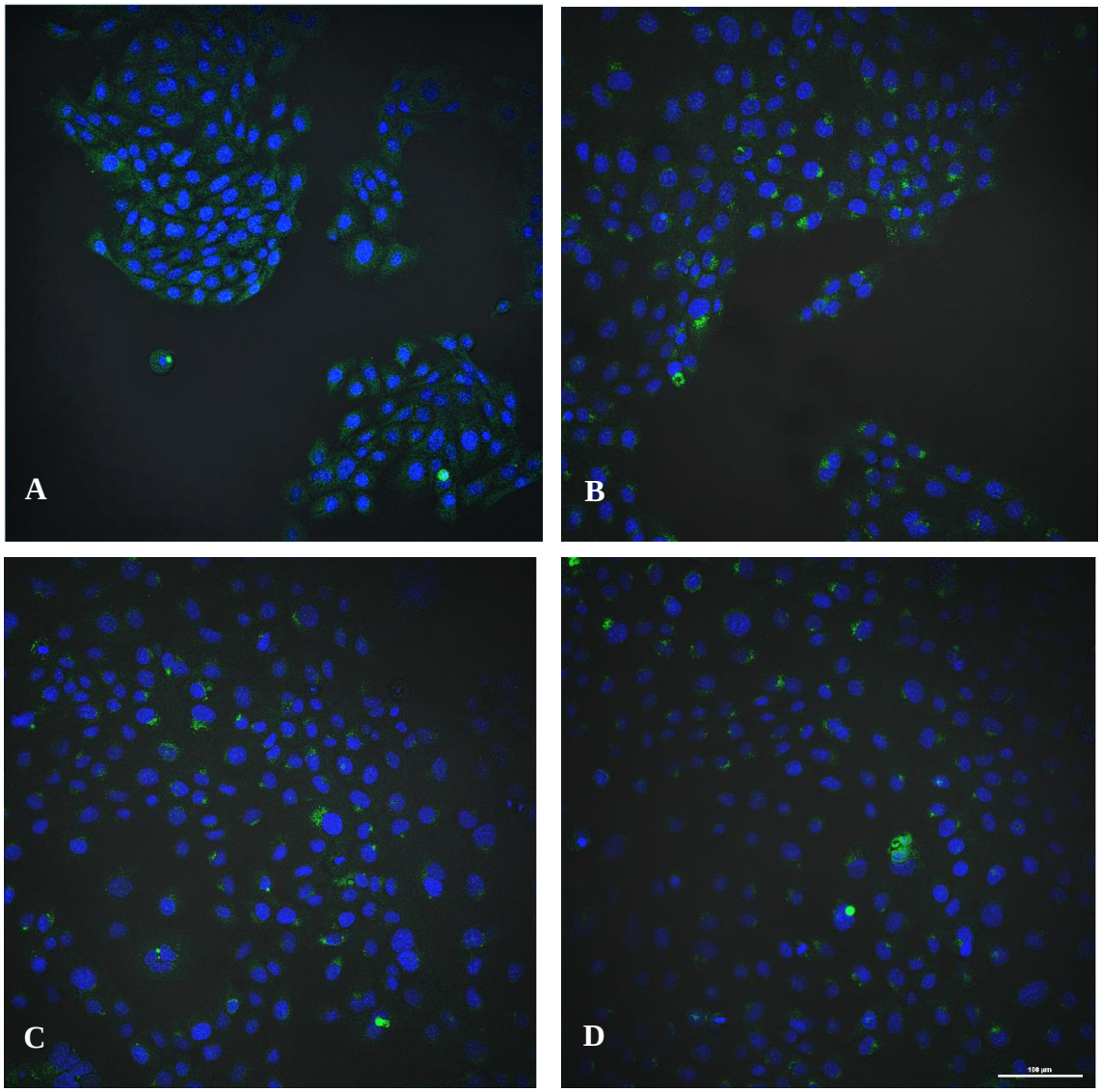

Figure 5-10 Images of immunocytochemistry for TAS2R38 in transiently transfected TR146 cells

TR146 cells were transiently transfected with cDNA coding for the human bitter taste receptor hTAS2R38. To visualize hTAS2R38 proteins (green) a hTAS2R38 specific antibody was used with a FITC conjugated secondary antibody. The cell nuclei were labelled with Hoechst (blue). Overlay pictures of the green and blue channels are shown to show the levels of receptor expressed at the plasma membrane. Scale bar represents 100µm. Representative image shows fluorescence in TAS2R38 transfected TR146 cells (A.) and secondary antibody only control (B.). Representative image of fluorescence in mock-transfected TR146 cells (B.) and secondary antibody only control (D.) also shown.

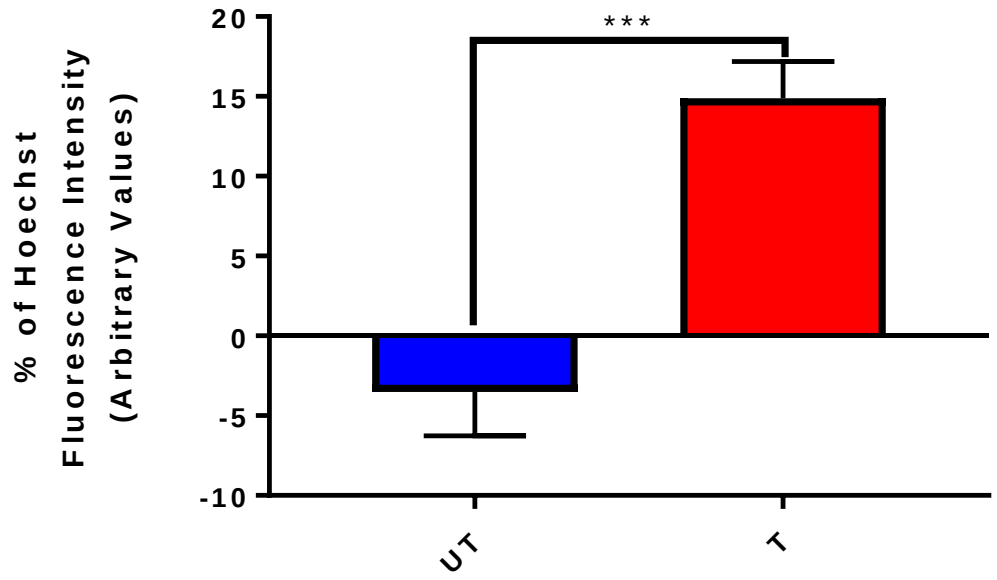

Figure 5-11 Quantification of TAS2R38 protein expression in transiently transfected TR146 cells determined by immunocytochemistry

Mean (+/-SD) hTAS2R38 protein expression determined by immunocytochemistry. TR146 cells were transiently transfected with cDNA coding for the human bitter taste receptor hTAS2R38. To visualize hTAS2R38 proteins a hTAS2R38 specific antibody was used with a FITC conjugated secondary antibody. The cell nuclei were labelled with Hoechst. Relative fluorescence intensity of hTAS2R38 expressed as a percentage of Hoechst fluorescence intensity. Intensity values corrected for background fluorescence, determined as intensity of FITC staining without TAS2R38 primary antibody. Statistical significance determined using independent student's T test. **P=<0.01. Data represent n = 2 (4-6 analyses).

Immunocytochemistry, using a TAS2R38 specific antibody, was used to further confirm protein expression of TAS2R38 in transiently transfected TR146 cells. Significantly greater TAS2R38 staining was seen in transfected cells, with a mean (+/-SEM) relative fluorescence of 14.9 +/-2.3 compared to -3.5 +/- 2.7 (arbitrary values) in un-transfected cells (Figure 5-11). There was a high level of background staining seen in cells treated with secondary antibody only, however the level of TAS2R38 specific staining was still higher in transfected cells when corrected for background fluorescence (Figure 5-10).

5.3.11 Characterisation of Endogenous Taste Receptor Expression in SCC090 Cells

Expression of TAS2Rs, including TAS2R38, was also demonstrated in SCC090 cells, as an alternative model to the transfected TR146/TR146 MUC1 cell lines, characterised above. Three bitter receptors, namely TAS2R10, TAS2R7 and TAS2R43 were chosen to demonstrate bitter taste receptor expression in SCC090 cells, as they represent promiscuous receptors which can be activated by common bitter compounds. All three chosen receptors can be activated by caffeine, which was used as a representative bitter taste in the volunteer study.

5.3.12 Analysis of Human Bitter Taste Receptor mRNA Expression in SCC090 Cells

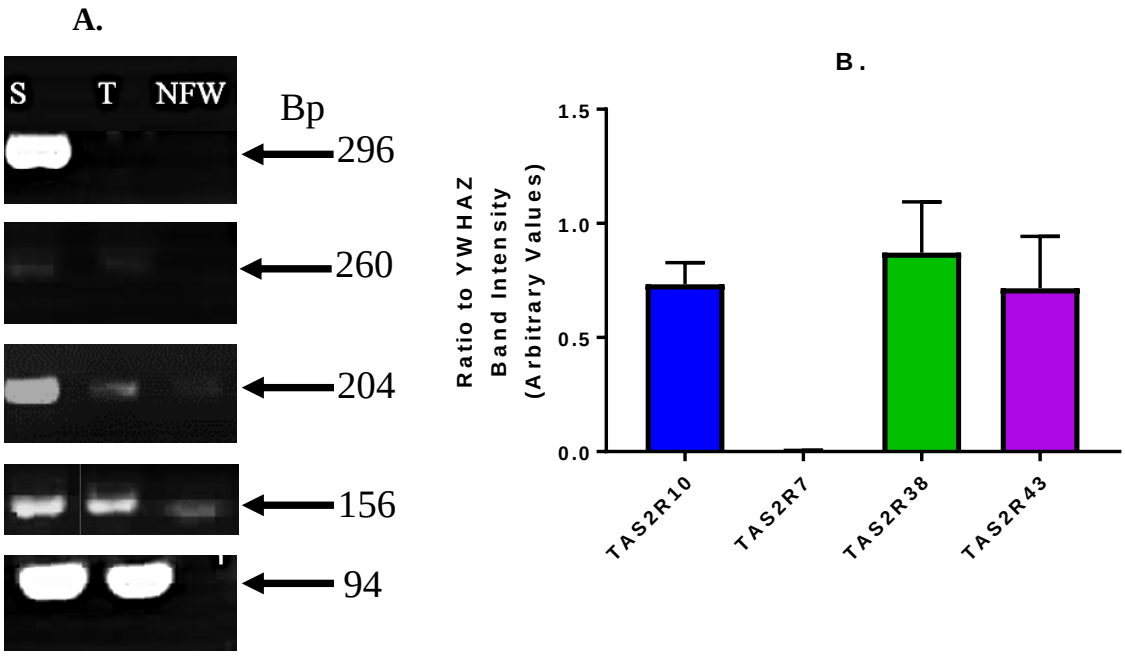

Figure 5-12 RT-PCR of TAS2R receptors expressed in SCC090 cells
mRNA from SCC090 cells were used for RT-PCR of TAS2R bitter receptors. TR146 cell mRNA used as negative control. (A) Representative image of agarose gel of PCR products using TAS2R10 (296Bp), TAS2R7 (260Bp), TAS2R38 (204Bp) and TAS2R43 (156Bp) primers. YWHAZ housekeeping gene used as cDNA control (94Bp). S=SCC090 (duplicate samples), T=TR146, NFW=nuclease free water (negative control). (B) Semi quantification of bitter taste receptor gene expression in SCC090 cells. Mean (+/-SEM) band intensity of agarose gel of PCR products determined using Image J software. Mean (+/-SEM) band intensity of gene of interest expressed as a ratio to YWHAZ housekeeping gene (94Bp). Data representative of n = 2-3 (2-4 analyses).

Expression of TAS2R38 and of 3 other bitter receptors, namely TAS2R10, TAS2R7 and TAS2R43, in SCC090 cells was investigated by RT-PCR. TR146 cells were used as a control as expression levels could be compared to those demonstrated in the microarray. mRNA encoding TAS2R38, TAS2R10 or TAS2R43 was present in SCC090 cells, but not in TR146 cells (Figure 5-12, lane S and T respectively). For TAS2R7, no amplification product was evident in either cell line. Amplification of TAS2R43 was less reproducible (seen in 2 out of 3 analyses) and occurred in TR146 cells. However, data from the microarray of TR146 RNA demonstrated a lower expression value for TAS2R43 than for TAS2R10, for which no amplification product was evident in RT-PCR (lane T, 296 bp).

RT-PCR analyses were also used to determine expression of sweet and umami taste receptors, T2R1, T1R2 and T1R3, in SCC090 cells. On agarose gel electrophoresis, no amplification products were observed (Appendix 16. Expression of Sweet and Umami Taste Receptors in SCC090 and TR146 Cells. Although in the absence of a positive control, no definitive conclusion regarding expression of these receptors could be made.

5.3.13 Characterisation of TAS2R38 Protein Expression in SCC090 Cells

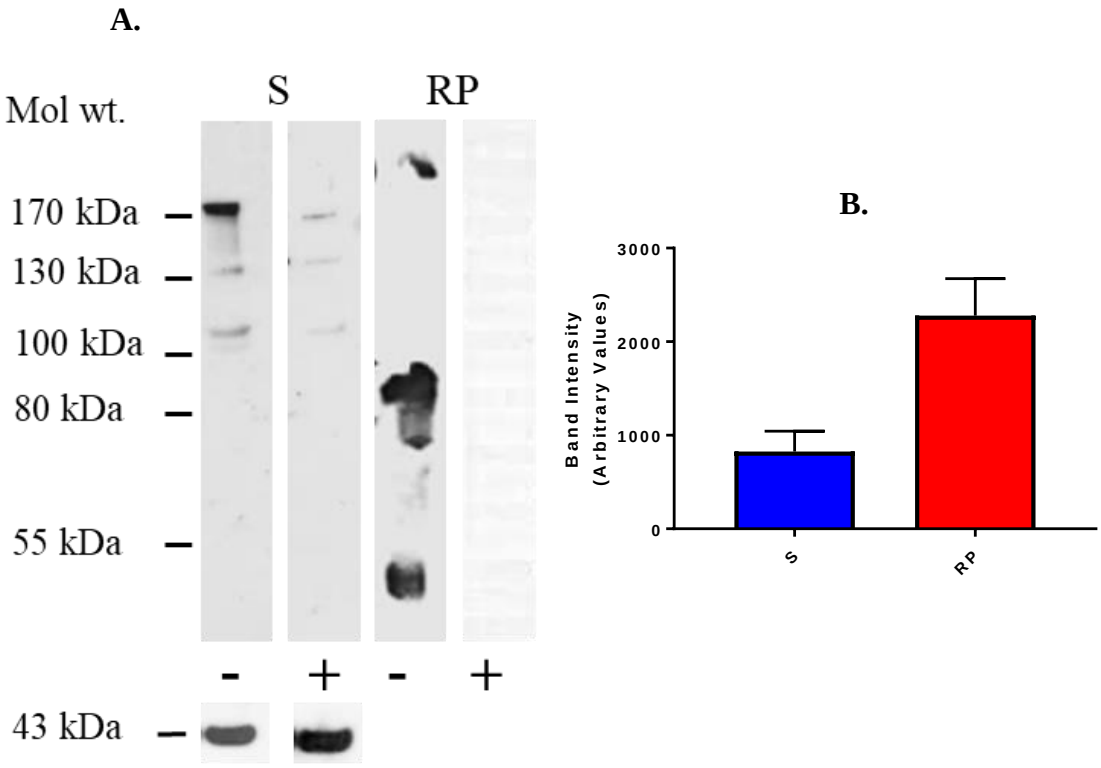

Figure 5-13 Immuno-blotting of SCC090 whole cell lysates visualized by staining with anti-TAS2R38 antibody.

Immunising peptide against TAS2R38 was used to determine antibody specificity. A. Representative immuno-blot image of TAS2R38 in SCC090 (S) cells shown without (-) and with (+) immunising peptide added to the primary antibody to block non-specific binding. Re-probing of the same blots with beta actin was used as a loading control =43kDa. RP=TAS2R38 recombinant protein (used as positive control). B. Mean (+/- SEM) band intensity of TAS2R38 in SCC090 cells (S) and TAS2R38 recombinant protein (RP). Intensity values adjusted for non-specific binding by removing the band intensity from immunising peptide blocked blots. Data represent n = 2-3.

SCC090 cells were also characterised as a potential model for determination of TAS2R38 responses to tastants. To determine protein expression of bitter taste receptors, immune-blotting was conducted using a TAS2R38 specific anti-body. As shown above for transfected TR146 cells, a band of high molecular weight (>170kDa) was detected in the cell lysate together with a less prominent band of approximate molecular weight 130kDa (Figure 5-13 lane S). These likely correspond to oligomeric forms of the receptor. A lower molecular weight band of approximately 45kDa, likely corresponding to the monomeric form of the N-glycosylated receptor, was also evident in the recombinant TAS2R38 preparation used as a positive control (lane RP). To show specificity of the bands, a titration was performed, and reduced intensity of both bands was seen with decreasing total protein. Mean (+/-SEM) band intensity in SCC090 cells was 825.2 +/- 218.9 compared to 2280 +/- 393.8 in the recombinant protein. In addition, the bands of TAS2R38 were reduced in SCC090 and completely disappeared in the recombinant protein following peptide blocking, again demonstrating relative specificity of the TAS2R38 antibody.

5.3.14 Functional Characterisation of TAS2R Expression in TR146 MUC1 and SCC090 Cells

TR146 MUC1

As transfection efficiencies were similar in TR146 and TR146 MUC1 cells, the MUC1 expressing cell line was chosen to conduct functional experiments of TAS2R38, as over-expression of MUC1 may facilitate improved muco-adhesion of salivary mucin. With the overarching aim of the project being to investigate the effect of the salivary pellicle on TAS2R38 responses, the MUC1 cell line was therefore deemed more suitable for use in response assays.

To demonstrate functionality in TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells, calcium responses to TAS2R38 agonist, PTC, were investigated. Cells were transfected with a 2:1 TAS2R38:Gα16Gust44 cDNA ratio and 0.3µl transfection reagent. This was in line with optimisation experiments, showing increased intracellular calcium responses to 25µM PTC, under these transfection conditions (Appendix 17. Optimisation of TAS2R38:Gα16Gust44 Functional Expression in Transfected TR146 MUC1 Cells). In addition, the 2:1 cDNA ratio yielded comparable mRNA expression of TAS2R38 and Gα16Gust44, in semi-quantified RT-PCR (Figure 5-5) and no significant differences were shown between different transfection ratios for protein expression, in western blotting of TAS2R38 (Figure 5-8).

Responses to concentrations of PTC, ranging from 25 to 100µM, elicited an intracellular calcium response in co-transfected TR146 MUC1 cells significantly greater than that seen in mock transfected cells. An average response to PTC of 0.31 +/- 0.09 was seen in TAS2R38:Gα16Gust44 transfected cells, compared to 0.16 +/- 0.04 in mock transfected cells (mean +/-SEM peak fluorescence 340nm:380nm ratio, arbitrary values) (Figure 5-14). Concentrations of 25, 50, 100 and 200µM PTC elicited a response greater than the carrier control in TAS2R38:Gα16Gust44 transfected cells (Figure 5-14). 100µM PTC elicited the greatest response in transfected cells, with a mean (+/-SEM) fluorescence intensity of 0.53 +/- 0.14 compared to 0.09 +/- 0.03 for carrier control only. Concentrations above 100µM elicited reduced responses, in a dose dependent manner. In mock transfected cells, none of the PTC concentrations elicited a response which was significantly greater than carrier control (Figure 5-14). To further

demonstrate TAS2R38 specificity of responses, similar results were shown for 1mM PROP (Appendix 18).

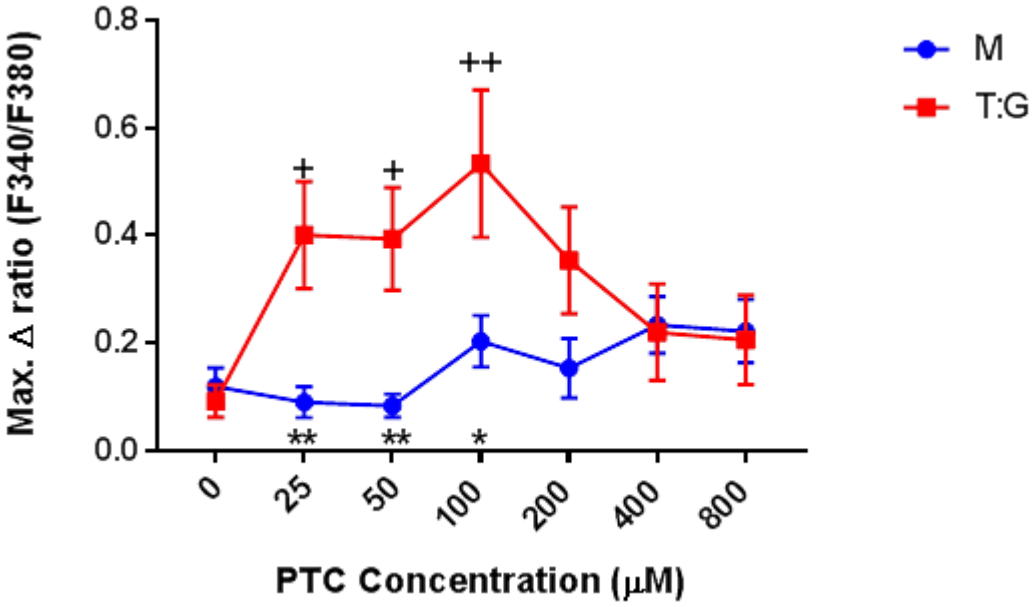

Figure 5-14 FLEX station measurement of intracellular calcium in TR146 MUC1 cells with PTC

Intracellular calcium changes in transfected TR146 MUC1 cells loaded with the fura2-am calcium indicator with increasing concentrations of PTC. FLEX station fluorescence plate reader was used to measure intracellular calcium response (iCa^{2+}). The panel shows mean (+/- SEM) peak response (fluorescence 340nm:380nm ratio) from baseline after compound addition, in mock transfected (M) and TAS2R38:Ga16Gust44 co-transfected (T:G) TR146 MUC1 cells. Data is representative of 3-4 experiments (12-15 analyses). Analysed for statistical significance using one-way ANOVA with Tukey's multiple comparisons. Significance = P value < 0.05 */+ P < 0.01 **/++ P < 0.001 ***/+++, P<0.0001 ****/++++. Analysed for statistical significance using students T test (difference between transfection conditions = *) and Kruskal-Wallis test with Dunn's multiple comparisons (T:G) or one-way ANOVA with Dunnett's multiple comparisons (M) (difference from baseline within each transfection condition = +).

SCC090

Since RT-PCR analyses indicated expression of TAS2R10 and TAS2R38 in SCC090 cells and western blotting also confirmed expression of TAS2R38, stimulation of calcium responses by agonists of these receptors was investigated to demonstrate functional expression. Caffeine was selected as an agonist for TAS2R10 since it was also used in the volunteer study in chapter 3. This would therefore provide a model for caffeine taste responses, which would be compared to the *in vivo* responses shown in the volunteer study. Both PTC (Figure 5-15 A) and PROP (data shown in Appendix 18. Calcium responses to PROP in SCC090 and TR146 MUC1 cells) were used to as TAS2R38 agonists. Intracellular calcium responses to 0.16 and 0.31 and 0.63mM caffeine were significantly higher than responses to the carrier buffer alone with mean (+/-SEM) fluorescence intensity values of 14.62 +/-2.78, 14.44 +/-3.04 and 9.18 +/-1.64 (arbitrary values) respectively, compared to 1.76 +/-0.83 in the carrier control (Figure 5-15 B). At concentrations of caffeine of 1.25mM and above, responses were reduced in a dose dependent manner. Responses to PTC were shown using 25 and 200µM PTC that were significantly greater, 20.74 +/-6.92 and 15.95 +/-2.59 respectively, compared to 4.54 +/-1.72 for the carrier control (Figure 5-15 A).

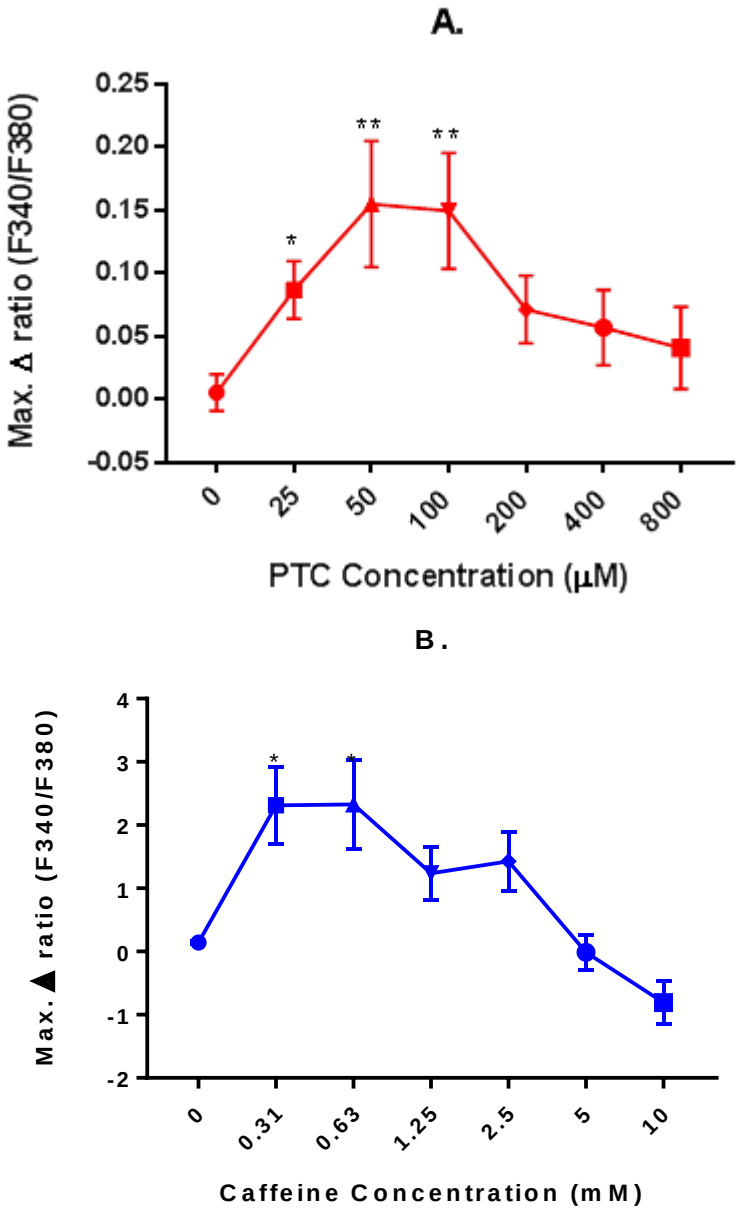

Figure 5-15 FLEX station measurement of intracellular calcium in SCC090 cells with bitter taste compounds

SCC090 cells were loaded with the fura2-am calcium indicator and fluorescence emissions recorded before and after exposure of the cells to increasing concentrations of bitter receptor agonists. FLEX station fluorescence plate reader was used to measure intracellular calcium response (iCa^{2+}). The panels show mean (+/- SEM) peak response (fluorescence ratio 340nm:380nm) from baseline after addition of compound, PTC (TAS2R38 agonist, A.) and caffeine (TAS2R10 agonist, B.). Data is representative of 3-4 experiments (9-12 analyses). Analysed for statistical significance using Kruskal-Wallis test with Dunn's multiple comparisons (A) or one-way ANOVA with Tukey's multiple comparisons test (B). Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****.

5.4 Discussion

5.4.1 Expression of Taste Receptors in TR146 cells

TAS2Rs are expressed on the tongue, in the oral cavity, and by several extra-oral cell types (Deshpande et al. 2010, Wu et al. 2002, Rozengurt et al. 2006, Wölfle et al. 2015). To study the activation of these receptors in response to bitter taste compounds, over-expression systems have been used widely, whereby taste receptor cDNA is transiently transfected into non-taste cells (Brockhoff et al. 2007, Bufe et al. 2002, Tsien et al. 1985, Meyerhof et al. 2010). To date, there are no such studies which have used a cell line relevant to the oral cavity. Moreover, a layer of saliva constantly bathes taste buds on the tongue, providing a peri-receptor milieu, which may affect tastant mediated responses, of taste receptors (Matsuo 2000). However, the effect of saliva on transduction of taste molecules to the taste receptors is rarely considered in these models. Therefore, the relevance of such cell-based over-expression systems, to oral taste transduction *in vivo*, may be limited. In this study, TR146 cells, which have previously been used as a model of the oral epithelium, were used as a biologically relevant model of taste receptor activation, in the oral cavity.

Since TR146 cells were to be used as a model for taste, endogenous expression of taste receptors was first determined. Microarray analysis of TR146 cell RNA showed low level expression of 14 TAS2R bitter receptors along with the sweet and umami taste receptors which were expressed at levels of 2.55 (TAS1R1, 2.71 (TAS1R2) and 2.55 (TAS1R3). RNA expression levels of all genes ranged from 12.79, for NADH dehydrogenase 2, to 1.29 for UDP glucuronosyltransferase 2 family, polypeptide B17 (UGT2B17). Expression of multiple G proteins was also shown, including GNAT3 and GNB1 which combine with GNG13 to form gustducin *in vivo*.

To observe the effect of treatment with a bitter taste compound on expression of receptors, TR146 cells were treated with PTC, and microarray analysis was performed on RNA from treated and un-treated cells. Expression of GPCRs may be regulated by their agonists (Zhang et al. 2015). Therefore, it was anticipated that PTC treatment might lead to up or down regulation of TAS2R38, or another receptor which may be involved in TAS2R independent responses to PTC. Following PTC treatment, the expression of TAS2R38 RNA was un-changed in TR146 cells. However, 61 genes were

up-regulated, and 109 genes were down-regulated following PTC stimulation. Of these, 10 genes could be associated with chemo-sensation in a search of the literature.

Interestingly, the orphan GPCR, GPR20, was over expressed in TR146 cells treated with PTC. This coincided with down regulation of another GPCR, melatonin receptor 1 (MT1). MT1 has been shown to couple to the orphan GPCR50 which induces a conformational change in MT1 and results in altered ligand binding (Levoye et al. 2006). Furthermore, MT1 homodimers are internalised upon ligand stimulation, while GPR50/MT1 heterodimers remain at the cell surface (Levoye et al. 2006). Additionally, co-expression of GPR50 and MT1 results in a reduction in expression of MT1 homodimers and an increase in expression of GPR50/MT1 heterodimers (Levoye et al. 2006). As shown in other GPCRs, it is possible that following ligand induced internalisation, MT1 expression is subsequently reduced, as the receptor may be subject to endocytosis and degradation in the lysosome (Koenig and Edwardson 1997, Gray and Roth 2001, Kallal et al. 1998). Like GPR50, GPR20 is an orphan receptor with no known ligand (Hase et al. 2008). It could be tentatively hypothesised therefore, that GPR20 couples to MT1 in the same way as GPR50. If PTC is indeed an agonist for GPR20/MT1, treatment may therefore induce up-regulation of GPR20 and down-regulation of MT1 homodimers. Heteromeric coupling of GPCRs alters ligand binding, by changing the conformation of the receptor binding site. It is possible that, only in the heteromeric state, can GPR20 and MT1 be activated by PTC. Additionally, MT1 has previously been shown to be activated by certain bitter tastants (Zubare-Samuelov et al. 2003, Mubashshir, Ahmed and Ovais 2011). MT2 expression has also been shown in the circumvallate taste tissue of rats (Zubare-Samuelov et al. 2003). Melatonin, an MT1 agonist, induces aversive taste responses in rats, similarly to bitter taste compounds (Zubare-Samuelov et al. 2003). This may indicate that tastants can stimulate non-taste receptors, which may have other biological effects, and conversely, non-taste receptors may form alternative taste response pathways.

However, the results from this study should be interpreted with caution since the microarray was only performed once. It is not known therefore, if the expression patterns seen would be reproducible. Furthermore, a total of 170 genes were either up or down regulated by PTC treatment, therefore it would be impossible to rule out involvement of other proteins or receptors without further research. Future work could be directed towards further characterising expression of both receptors and their co-

localisation, before and after PTC treatment. Additionally, over-expression models and/or chemical inhibition of GPR20 and MT1 could show their involvement in a PTC response pathway.

5.4.2 Optimisation of TAS2R38:Gα16Gust44 Over-Expression System in TR146/TR146 MUC1 Cells

As only low levels of TAS2R expression could be shown in TR146 cells, a heterologous expression system was created by transiently transfecting with TAS2R38 and Gα16Gust 44 cDNA. The aim of this was to induce over-expression of the receptor/G-protein in a biologically relevant cell line, oral epithelial cells, to produce a model for transduction of tastants to taste receptors. This model would eventually be used, with saliva samples from older and younger adults, to understand the effect of the mucosal pellicle on tastant diffusion. As such, optimisation was conducted in both wild type TR146 cells, and the stably expressing MUC1 cell line. TR146 MUC1 cells may be a better model for salivary muco-adhesion, since expression of MUC1 in oral epithelial cells facilitates binding of MUC5B through mucin-mucin interactions (Ployon et al. 2016, Pramanik et al. 2010). TAS2R38 was chosen as it is one of the most well studied bitter receptors and because there was a clear difference in perception of PTC between older and younger adults, shown in the volunteer study conducted in chapter 3.

To demonstrate the efficiency of transfection of TR146 and TR146 MUC1 cells, western blotting was conducted using a commercially available TAS2R38 specific antibody and an antibody directed to the FLAG tag on the Gα16Gust44 construct. Expression of TAS2R38 protein was detected in TAS2R38 transfected and TAS2R38:Gα16Gust44 co-transfected TR146 and TR146 MUC1 cells. The FLAG tag could also be detected in transfected TR146 MUC1 cells, implying protein expression of the Gα16Gust44 protein. The transfection efficiency was optimised by varying the cDNA concentrations used in transfections. Semi-quantification of western blot band intensities suggested that the double transfection gave rise to reduced TAS2R38 expression, compared to single transfection in TR146 cells, but not in TR146 MUC1 cells. There was significantly greater TAS2R38 protein in the TR146 cells transfected with 0.5 and 1µg of TAS2R38 cDNA, compared to mock transfected cells. In both cell lines, transfection with TAS2R38 or TAS2R38:Gα16Gust44 led to increased, although not statistically significant, TAS2R38 protein in western blotting, compared to mock or un transfected cells. There was some, TAS2R38 expression seen in western blotting of

mock or un-transfected TR146 cells, however, this was not consistent in all blots. Meanwhile, TAS2R38 expression in TAS2R38 transfected cells was reliably shown in western blotting. This suggests that the transfection led to more consistent over-expression of TAS2R38. Similarly, levels of FLAG protein were greater in both Gα16Gust44 transfected and TAS2R38 co-transfected cells, compared to mock-transfected, but this was also not statistically significant.

While there was no statistically significant difference between cDNA concentrations used for transfection, in terms of protein seen in western blotting, transfection with 1µg TAS2R38 plasmid gave rise to the greatest, relative, levels of protein, in both cell lines. In the double transfection, a 1:1 TAS2R38:Gα16Gust44 ratio led to the greatest TAS2R38 protein in TR146 cells while in TR146 MUC1, 4:1 ratio appeared to be most efficient for TAS2R38 protein seen in western blotting. For Gα16Gust44 protein, 1µg cDNA and a 1:1 co-transfection ratio led to the highest levels of FLAG protein. The high variation in TAS2R38 and FLAG protein between western blots may be the reason for the lack of statistical significance.

Some TAS2R38 protein was seen in un- and mock-transfected cells in western blots. The bands could be partially blocked by the immunising peptide, suggesting some specificity. The band intensity was however, higher in transfected cells, even after subtracting the intensity of non-specific bands seen after blocking of TAS2R38 with the immunising peptide. Low level constitutive TAS2R38 expression may explain this. This would also be consistent with the low level of RNA expression shown in the micro-array of TR146 cells.

The FLAG antibody was relatively un-specific and gave rise to multiple bands at various sizes, detected using immuno-blot. Furthermore, transfection with 4µg TAS2R38 cDNA led to appearance of a FLAG tag band despite the TAS2R38 construct not being FLAG tagged. Cross reactivity with the TAS2R38 construct could not be ruled out and BLAST analysis of the flag sequence determined several human proteins which could give rise to cross-reaction, including a procollagen-lysine modifying enzyme, zinc finger proteins and dynamins (GTPases). A limitation of the experiment lies in the lack of positive control for the FLAG antibody. While a recombinant protein and commercially available over-expression lysate was used as a control in the TAS2R38 western blotting, this was not available for the FLAG tag and so there was a lack of optimisation of the antibody needed to demonstrate specificity.

Western blot analysis of TAS2R38 in TR146 and SCC090 cells proved difficult due to the formation of receptor oligomers which were not easily disrupted by SDS detergent. As such, the band sizes were variable between repeats of the experiment and between cell line. The expected molecular weight of the un-glycosylated TAS2R38 monomer is 38kDa and N-linked glycosylation would increase the molecular weight to approximately 44kDa. However, band sizes seen at approximately 80kDa and over 100kDa suggest presence of dimers and oligomers respectively. This is in agreement with Behrens et al, who also showed higher than expected molecular weight band size, and multiple band pattern of TAS2R38 on western blots (Behrens et al. 2012). Further, the commercially available recombinant protein, purified by anti-DDK immunoaffinity column, had a band at around 40kDa, likely a monomer and one at 80kDa, likely a dimer, which suggests formation of higher order oligomers occurs in situ and not in a purified protein. In a heterologous expression system, 90% of TAS2Rs, including TAS2R38, form oligomers (Kuhn et al. 2010). Formation of both homo- and hetero-logous oligomers, has been observed in TAS2Rs (Kuhn et al. 2010). Co-expression of different TAS2Rs has been shown to occur in taste cells *in vivo* (Behrens et al. 2007). Other GPCRs are also known to form hetero-oligomers *in vivo* (Kaupmann et al. 1998).

Oligomers may not be completely disrupted by SDS. SDS is a detergent which denatures proteins by forming aggregates at hydrophobic protein regions, un-folding the protein structure (Rath et al. 2009). The protein then takes on a helical conformation with random coils. Heating denatures the protein and SDS stabilises and maintains the unfolded form via charge repulsion, as SDS molecules bind to amino acid side chains (Shirahama, TSUJII and TAKAGI 1974). Transmembrane (TM) proteins have strongly hydrophobic residues within the membrane spanning domains, with few polar residues which undergo packing against similar sequences in nearby TM regions, through side chain−side chain hydrogen bonds and electrostatic interactions (Zhou et al. 2001). TAS2Rs have 7 TM spanning domains (Pierce, Premont and Lefkowitz 2002). The predominantly hydrophobic TM regions are tightly packed together because of hydrophobic interactions, prohibiting binding to SDS. Furthermore, the TM domain is hydrophobic and has an affinity for detergent, but the internal segments of the protein contains some polar residues which have a preference for aqueous solvent (Rath et al. 2009). In the cytosol, TM proteins fold in a manner which means that hydrophobic residues are hidden while polar or charged residues are exposed to solvent. As such, in SDS, a TM protein may be partially dissolved by detergent while the polar regions are

dissolved in the surrounding aqueous environment (Tulumello and Deber 2009). Therefore, full solubilisation of the protein, at the site of oligomerisation, is prevented by SDS and tertiary structures are not disrupted. As such, the observation of oligomers and dimers on TAS2R38 western blot of cell lysates is not un-expected. The immunising peptide was used to block specific binding of TAS2R38 and therefore identify bands corresponding to dimers and oligomers. After blocking with the peptide, bands at high molecular weight partially, or in some cases completely, disappeared suggesting TAS2R38 specificity of these bands. The bands at 55kDa and 38kDa were also eliminated by the immunising peptide, which indicates these are also likely to be TAS2R38 specific.

There was variation amongst transfection efficiency, seen as differences in protein and RNA levels, as well as in responses between replicates of the calcium assays. This variation could be due to differences in DNA uptake in individual cells or in cell cycle phase at the time of transfection (Zabner et al. 1995, Brunner et al. 2000). Transport of plasmid DNA to the cell nucleus occurs predominantly during mitosis. Plasmid DNA can be broken down in the cell cytoplasm as it is taken up by the nucleus (Lechardeur et al. 1999). Therefore, transfer of DNA to the nucleus will be higher in cells which are transfected in the late S phase (DNA replication) or the G2 phase (second growth) compared to those transfected during the G1 (first growth) or G0 (quiescence) phases. This is because the time between transfection and transport to the nucleus, during which the DNA can be degraded in the cytoplasm, is reduced. Synchronising the cell cycle before transfecting would therefore encourage greater and more homogenous gene expression (Roelse et al. 2018). Similarly, addition of nuclease inhibitors during transfection would also likely enhance the efficiency of gene expression, by minimising degradation of the plasmid (Ross et al. 1998). In this study, cell cycles were not controlled for, but expression of TAS2R38 and Gα16Gust44 was still demonstrated at protein and RNA level and was sufficient to yield function in calcium assays.

5.4.3 Analysis of Endogenous Expression of Taste Receptors in SCC090 Cells

In chapter 4, the tongue epithelial cell line, SCC090 was shown to express MUC1 and facilitate MUC5B binding from WMS. It was therefore proposed that this cell line might provide a useful model for taste transduction, as it originates from the human tongue and could be used to study the mucosal pellicle *in vitro*. To investigate endogenous expression of taste receptors in SCC090 cells, RT-PCR was conducted for a

panel of bitter receptors as well as the sweet and umami receptors. Amplification of fragments of TAS2R10, TAS2R38 and TAS2R43 were demonstrated in RT-PCR of RNA from SCC090 cells. No amplification of TAS1R1, 2 or 3 was shown and therefore sweet and umami receptors may not be expressed in this cell line. hTAS2R10 is a promiscuous receptor, which can be activated by over 30 bitter compounds including caffeine, quinine and denatonium benzoate, all frequently used in bitter taste testing studies (Meyerhof et al., 2010). TAS2R38 protein expression was also demonstrated in SCC090 cells, by western blot. Specifically, 2 high molecular weight bands were seen, indicating presence of TAS2R38 oligomers, and one band at 55kDa, suggesting TAS2R38 dimers.

The study was limited by the number of TAS2R genes that were screened in RT-PCR of SCC090 cells. It is possible that other bitter taste receptors are also constitutively expressed. Furthermore, only TAS2R38 protein expression was demonstrated, while expression of other TAS2Rs was shown only at mRNA level. Additionally, optimisation of the primers was not possible as a positive control was not available. A suitable control would have been an over-expression cell mRNA or plasmid cDNA for each TAS2R, along with a negative control: mRNA from a cell line that does not express TAS2Rs. As such, specificity of the primers was not confirmed, except for TAS2R38 for which the plasmid DNA was used (Appendix 19). However, all primer pairs were run through BLAST software to ensure they were unique to the gene of interest.

5.4.4 Functional Characterisation of TAS2R Receptor Expression in Human Oral Epithelial Cell Lines

To demonstrate that the TAS2R expression, shown in SCC090 cells and transfected TR146 MUC1 cells, was functional, intracellular calcium levels were measured in response to bitter taste compounds. Upon activation of TAS2R receptors, the coupled G protein is activated and this in turn activates PLCβ2. PLCβ2 hydrolyses PIP2 into DAG and IP_3 which activates IP_3R3 expressed by the endoplasmic reticulum. IP_3R3 facilitate calcium release from the ER stores and so, raised intracellular calcium is a measurable response to TAS2R stimulation.

For functional assays, the transfection conditions used were based on a preliminary assay showing increased calcium response to 25µM PTC, in cells transfected with 100ng TAS2R38, 50ng Gα16Gust44 and 0.3µl transfection reagent, compared to other

transfection conditions and mock-transfected cells. The calcium responses to PTC were also generally higher 24 hours following transfection, compared to 48 hours (data shown in Appendix 17). Observation of the cells indicated that viability was compromised 48 hours post transfection, although viability assays were not conducted to prove this. In western blotting and PCR of TAS2R38 and Gα16Gust44, there were no significant differences between cDNA amounts used in the co-transfection, for protein and mRNA expression levels. This is further supported by the findings of Thalmann et al., who showed that transfecting HEK293T cells with TAS2R38 in a concentration range of 37.5 to 150 ng produced effective functional expression and agonist induced calcium signals were not affected by cDNA amount used (Thalmann, Behrens and Meyerhof 2013).

Both SCC090 and TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells exhibited raised intracellular calcium, upon stimulation with PTC and PROP. There was a small, but insignificant, increase in intracellular calcium in mock-transfected TR146 MUC1 cells upon PTC stimulation. Certain bitter compounds can diffuse across the cell membrane, to activate G-proteins directly, in the absence of TAS2R channels. Furthermore, as discussed above, other receptors may be present in TR146 cells which can be activated by PTC, leading to a calcium response. The lack of significant calcium responses in mock transfected TR146 MUC1 cells suggests that, while low level constitutive expression of TAS2R38 was apparent in the microarray and western blotting of this cell line, this was not sufficient to elicit receptor functionality.

Initial functional experiments were conducted in both TR146 and TR146 MUC1 cells. However, since there were no noticeable differences in responses between the 2 cell lines, and successful transfection could be shown in both, the MUC1 cell line was selected for further study since it provides a good model for muco-adhesion. This was a requirement of the cell model, since the ultimate aim was to use saliva samples to investigate their effect on TAS2R38 responses.

In both SCC090 and transfected TR146 MUC1 cells, PTC stimulation led to increased intracellular calcium at concentrations between 25 and 100μM. At higher concentrations, 200-800μM, the response diminished in a dose dependent manner. It is not clear why the concentration curve displayed this pattern and explanations are merely speculative without further testing, such as viability assays.

Responses to PTC concentrations between 25-100µM may represent TAS2R38 mediated calcium release. This was in line with previous studies, which showed TAS2R38 independent calcium responses to PTC at concentrations over 100µM, in cells which do not express TAS2R38 (Meyerhof et al. 2010). In mock transfected TR146 MUC1 cells, there was a slight, although not statistically significant, rise in intracellular calcium with 100µM PTC which further supports the idea of TAS2R independent responses at these concentrations.

There was a reduced response to 200-800µM PTC in TR146 MUC1 and SCC090 cells, compared to the response to 25-100µM PTC. It is possible therefore that higher concentrations of PTC are toxic to the cells. Indeed, bitter taste is thought to be a survival mechanism for protection against poisons. PTC is highly bitter in those with the taster form of the TAS2R38 gene, so it would not be surprising for PTC to be toxic at high doses. However, previous studies looking at toxicity of PTC on mammalian cells have shown only modest reduction in cell viability, with PTC concentrations much higher than those used in this study (>10mM). Although this was shown in hepatic cells which have high capacity for detoxification (Elia et al. 1994). Furthermore, high concentrations of PTC did not affect mock transfected TR146 MUC1 cells which suggests that the effect is confined to TAS2R38 responses.

It is also possible that the agonist causes internalisation and even down-regulation of the TAS2R38 receptor, in a concentration dependent manner. Certainly, agonist induced internalisation has been shown in some TAS2Rs and in other GPCRs and provides a mechanism for de-sensitisation following agonist stimulation (Robinett et al. 2011, Meyerhof et al. 2005, Bufe et al. 2002, Koenig and Edwardson 1997, Gray and Roth 2001, Kallal et al. 1998). It is also possible that receptor saturation occurs at concentrations above 100µM, but in that case a plateau of the response would be expected. Furthermore, PTC is poorly soluble in water and higher concentrations of PTC may be less well solubilised, rendering the compound incapable of receptor activation, upon application to the cells. Dissolving in DMSO can improve solubility of PTC, but DMSO also stimulates a calcium response in mammalian cells and may even be a weak agonist of TAS2R38 (Verbeurgt et al. 2017). As such, DMSO was not added to the agonist solution since it could produce non-specific responses.

The response seen in transfected TR146 MUC1 cells was significantly greater than in mock-transfected cells, which further supports the suggestion of successful transfection.

Additionally, in the double TAS2R38:Gα16Gust44 transfected cells, responses were significantly higher than in mock transfected. Meanwhile, a single transfection with either TAS2R38 or Gα16Gust44 did not lead to higher responses than a mock transfection (data shown in Appendix 10). This confirms the importance for gustducin in TAS2R38 mediated responses to bitter compounds and provides support for using a heterologous expression system when creating a model for taste. This also corroborates findings from previous studies, showing that bitter taste responses are significantly reduced in the absence of gustducin (Caicedo et al. 2003, Ruiz-Avila et al. 2001).

As expression of TAS2R10 was demonstrated in RT-PCR of SCC090 RNA, caffeine was also used in functional testing of this cell line. TAS2R10 is a promiscuous receptor which can be activated by caffeine among other bitter compounds (Meyerhof et al. 2010). SCC090 cells displayed increased intracellular calcium in response to caffeine which was significantly greater than the response to the carrier control only. At the higher end of the concentration range, the responses began to diminish which could be due to toxicity of the compound at high concentrations. Previous studies in Chinese hamster ovary (CHO) cells have shown that cell viability is reduced by concentrations of caffeine as low as 0.4mM, in a dose dependent manner. 4mM caffeine led to almost complete toxicity (Rayburn, Bouma and Northcott 2001). Furthermore, caffeine has been shown to induce apoptosis in mammalian cells at concentrations of 5mM and above (Fernandez, Lopez and Santa- Maria 2003).

The functional results presented here could be strengthened by use of a specific inhibitor for TAS2R38/10 and/or an siRNA knockout of the receptor gene(s) in SCC090 cells. To date, only one TAS2R38 inhibitor has been discovered (Greene et al. 2011). Probenecid was shown to be a complete, allosteric inhibitor of TAS2R38 calcium responses to PTC and PROP, in HEK293T over-expression systems and in JEG3 cells (Wölfle et al. 2016, Greene et al. 2011). However, prior to the discovery that probenecid blocks TAS2R38 functional activity, it was commonly used in calcium response assays, as it prevents cellular leakage of the calcium indicator dye. Functional activity of TAS2R38, expressed in HEK293T cells, was shown previously, even when probenecid was used to retain the calcium dye (Meyerhof et al. 2010). In preliminary experiments for this study, probenecid was used to inhibit TAS2R38 responses with variable results (data not shown). As a precaution, in this study probenecid was therefore only used as a calcium dye retainer in the assay for caffeine responses although, its specificity as a

TAS2R38 inhibitor requires confirmation. However, mock transfected cells were used as a control in the TAS2R38 experiments, and calcium responses to PTC/PROP were significantly lower than in TAS2R38 transfected cells, which supports the conclusion that those responses arose from TAS2R38 activation.

Many TAS2Rs, including TAS2R10, are promiscuous receptors and can be activated by multiple bitter compounds (Meyerhof et al. 2010). Conversely, many bitter compounds, including caffeine, can activate more than one TAS2R. Therefore, the identification of specific inhibitors of these TAS2Rs remains a challenge. It is entirely possible that the calcium responses seen in SCC090 cells were due to presence of other TAS2Rs or even TAS2R independent pathways. TAS2R43, which was shown to be expressed by SCC090 cells at mRNA level, has previously shown to be activated by caffeine (Suess et al. 2016).

5.5 Concluding Remarks

To create an effective *in vitro* model of taste that can be applied to testing the effects of age-related changes in saliva on taste loss, taste receptor expression was characterised in two oral epithelial cell lines, both of which were previously shown to facilitate salivary mucin binding. There was little evidence of constitutive expression of active taste receptors in TR146 cells and so, an over-expression system was created using cDNA for TAS2R38 and Gα16Gust44, as a substitute for gustducin which mediates taste responses *in vitro*. In SCC090 cells, derived from the human tongue, constitutive RNA expression of several bitter taste receptors was identified. Protein and mRNA expression of TAS2R38 was shown in both transfected TR146/TR146 MUC1 cells, and in SCC090 cells. TAS2R38:Gα16Gust44 co-transfected TR146 MUC1 cells displayed intracellular calcium responses to PTC and PROP which were not seen in mock transfected cells. This suggests that the transfection was successful, and that the subsequently induced over-expression of TAS2R38 was functional. Gα16Gust44 co-expression was vital for functionality of TAS2R38. SCC090 cells responded, with increased intracellular calcium, to caffeine and PTC in a dose dependent manner. To see the effect of saliva on transduction of bitter tastants to TAS2R receptors, the models developed in this chapter was then used with saliva samples in the next chapter, to mimic the salivary mucosal pellicle. This allowed an investigation of how changes in physical properties of saliva in ageing might affect taste function in older adults.

6 Utilising Cell Models of Saliva-Taste Receptor Interactions to Study the Effect of Age-Related Changes in Salivary Physical Properties on Taste

6.1 Introduction

Previous studies showing tastant-receptor interactions using *in vitro* heterologous expression models, have failed to consider the effect of saliva. Saliva plays an important role in taste function since xerostomic and dry mouth is frequently coupled with reduced taste sensitivity (Hershkovich and Nagler 2004, Negoro et al. 2004, Temmel et al. 2005). In addition, morphological changes in taste buds, coupled with reduced taste sensation, has been observed in salivary gland excised rats (Matsuo et al., 1997; Nanda and Catalanotto, 1981). The taste buds are constantly bathed in whole saliva, or in the case of the posterior tongue, in secretions from the von Ebner salivary glands (Gurkan and Bradley 1987, Gurkan and Bradley 1988). Saliva solubilises tastants and transports them to the taste buds where they can activate taste receptors (Pedersen et al. 2002, Bradley et al. 2003, De Almeida et al. 2008). Tastants in solution can only access taste receptors via diffusion through the salivary mucosal pellicle on the tongue (DeSimone and Heck 1980, Matsuo 2000). For this reason, the model created in this study, utilised cell lines originating from the human oral cavity. Both cell lines were shown, in chapter 4, to facilitate salivary MUC5B binding, similarly to the oral epithelium *in vivo*. It was also shown, in chapter 5, that transfection of TR146 MUC1 cells, with TAS2R38 and Gα16Gust44 cDNA, resulted in a functional model for the study of tastant-receptor interactions. Furthermore, SCC090 cells provide an alternative model of taste receptor function, since they were shown to constitutively express several TAS2R receptors, required for bitter taste function. Taken together, these results suggest that both cell lines could provide effective models to study the effect of saliva on taste receptor activation.

Water molecules in saliva interact with tastant molecules and this affects ion transfer, which initiates taste response, across the receptor cell membrane since water mobility is required for ion transfer to occur (Mathlouthi and Seuvre 1988). If a tastant has hydrophobic regions, hydrogen bonding with water molecules is restricted and as such, hydrophobic groups repel the water and are driven into the non-polar environment of the receptor membrane (Gardner 1978, Wiet and Miller 1997). Increased

hydrophobicity of a compound leads to reduced ability to form hydrogen bonds with aqueous saliva and thus the ability to reach taste receptors may be enhanced (Gardner 1978). For this reason, the structure of bitter compounds likely affects the way in which they can diffuse through the salivary pellicle to the taste buds.

Lipophilic compounds, for example caffeine, interact with mucins via hydrophobic and Van der Waal's interactions (Larhed et al. 1997, Norris and Sinko 1997, Matthes et al. 1992). Furthermore, positively charged low-molecular weight compounds, such as bitter tastants, or ions, such as salt, may bind to the negatively charged regions in mucin via electrostatic interactions (Winne and Verheyen 1990). Binding of lipophilic or ionic taste compounds to salivary mucin may perpetuate taste responses, by retaining compounds in the oral cavity for longer (Cook et al. 2017). Alternatively, an inhibitory action may occur by binding of mucin to taste compounds and removing them in the saliva (Sigurdsson et al. 2013). Further, the hydrophobicity of a compound may also affect its ability to diffuse through saliva as hydrophobic interactions occur with the hydrophobic regions of mucin molecules (Larhed et al. 1997). Small molecule, polar compounds may diffuse more easily because mucin has a mesh like structure with aqueous fluid between the structural components creating a pore like system (Lai et al. 2010, Lai et al. 2011). Assuming there is no interaction between the compound and mucin fibres, low molecular weight compounds pass through these pores unhindered while diffusion of higher molecular weight compounds is reduced. Where there are interactions between the compound and mucin, larger molecules may pass more easily than smaller molecules which can become trapped in the mucin network (Lafitte et al. 2007). Indeed, lipophilic compounds can also passively diffuse into epithelial cells via interaction with the lipid bilayer (Camenisch et al. 1998), providing a mechanism for direct activation of G-proteins exhibited by certain bitter taste compounds (Koyama and Kurihara 1972).

The results from this study demonstrated that ageing results in changes in the physical properties of saliva, including reduced viscoelasticity. This is likely because of alteration in mucin composition, with reduced total levels of MUC7 and impaired sialylation as shown in chapters 3 and 4. In addition, muco-adhesive properties of saliva from older adults were reduced, with lower binding of MUC5B to TR146 MUC1 cells. Muco-adhesion of saliva could play a role in retention of tastants on the tongue, which may affect taste receptor activation (Cook et al., 2017). Some studies have shown that

certain muco-adhesive polysaccharides can prolong the perception of taste molecules, perhaps because tastants are retained in the mouth, with access to taste receptors, for longer (Gallardo-Escamilla et al. 2007, Malkki et al. 1993, Cook et al. 2018). The observed changes in the saliva of older adults could therefore impact upon taste function, including the ability to taste bitter taste compounds. Bitter tastants are generally hydrophobic or lipophilic in nature and therefore their ability to activate taste receptors may depend on interactions with saliva. Previous studies have shown a general reduction in bitter taste sensation in older adults (Methven et al. 2012). In this study, perceptions of PTC were reduced in older adults, compared to younger. Therefore, saliva samples from older and younger adults were used to create a mucosal "pellicle" on top of the cultured oral epithelial cells, to observe how age-related changes in saliva might lead to altered tastant-receptor interactions.

This chapter describes use of the cell-models of taste, developed in chapter 5, to study the effects of saliva on transduction of taste molecules to the receptor. The effect of ageing on saliva was characterised in previous chapters and so in this chapter, saliva samples from older and younger adults were used to investigate how these changes might impact upon tastant-receptor interactions. TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells and SCC090 cells were incubated with saliva and intracellular calcium responses were measured following addition of PTC or caffeine, respectively. Since both cell lines were shown to bind salivary mucin in chapter 4, it was proposed that this would mimic the effect of the mucosal pellicle, on tastant-receptor interaction, occurring *in vivo*.

6.2 Methods and Materials

6.2.1 Cell Culture

Experiments were carried out using the TR146 MUC1and SCC090 cell lines, cultured as previously described in chapter 2.

6.2.2 Transfection of TR146 and TR146/MUC1 cells

To encourage over-expression of TAS2R38/Gα16Gust44, TR146/MUC1 cells were transfected using plasmid DNA prepared in chapter 5. The method for transfection is described in full, in chapter 2. A mock transfection was conducted alongside transfection using DNA of interest as a negative control, using empty pcDNA 3.1+ vector (Invitrogen, Carlsbad, CA, USA) as described in chapter 2.

6.2.3 Preparation of Whole Mouth Saliva Samples

Unstimulated whole mouth saliva (UWMS) samples, collected in chapter 3 of this study, were used to create a layer of saliva on top of cultured cells. Saliva samples were first clarified by centrifugation at 2000G for 10 minutes at 4°C. Samples were stored on ice during preparation and prior to addition to cell plates. To optimise the model for use with saliva, UWMS was collected from a single, healthy donor (27 years old). Saliva from this donor was used to normalise between cell plates during the assay of older and younger UWMS.

6.2.4 FLEX Station Intracellular Calcium Measurements

Intracellular calcium measurements were conducted using fluo-4 staining of cells, as described in chapter 2. Excess dye solution was removed, and fresh saline solution with whole mouth saliva was added (1:1 dilution, 50% WMS final dilution). Plates were incubated again for 15 minutes at 37°C before baseline fluorescence readings (excitation 480nm/emission 520nm) were taken for 1 second using the FlexStation 3 (Molecular Devices, San Jose, CA, USA). The automatic pipetting system on the FLEX station was set to add the taste compound after 30 seconds of plate reading and readings were taken every 5 seconds for 1-minute following compound addition. Ionomycin (10uM) was added after addition of the taste compound, as a control to elicit maximum response in cells and allow for normalisation between wells, and readings continued for a further 30 seconds. Data was analysed using Softmax Pro software and expressed as

the difference between maximum fluorescence and baseline fluorescence (normalised to ionomycin response).

As a control, every cell plate contained wells which had no WMS added and responses to saline solution with and without the bitter compound, were measured as described above. Wells were also reserved, on every plate, for a control UWMS sample from a single healthy donor (27 years old) to normalise responses between plates. For TR146 MUC1 assays, each cell plate also had wells of mock transfected cells, as a negative control. This allowed transfection efficiency to be determined, as the difference in response to the agonist between mock and TAS2R38:Gα16Gust44 transfected cells.

As discussed previously, probenecid (Sigma, Dorset, UK) was added to the fluorescent dye/saline mixture for measurement of responses to caffeine, to prevent leakage of calcium indicator dye. Probenecid was not used in PTC experiments due to the reported allosteric inhibitory action of probenecid on TAS2R38 receptors (Greene et al. 2011) and variability in responses to PTC with probenecid use, shown in this study. Some dye leakage was observed in cells where probenecid was not added, with resulting reductions in the fluorescence readings.

6.2.5 Statistics

Microsoft Excel (Version 1804, Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA) was used for data analysis and generation of graphs. The data was tested for normal distribution using the D'Agostino & Shapiro-Wilk normality tests. Data which was not normally distributed was analysed using non-parametric tests, Friedman test with Dunn's multiple comparisons (comparing 2 or more groups) and Mann Whitney U Test (comparing between 2 groups only). Data which was normally distributed was analysed with one-way ANOVA and Dunnett's multiple comparisons test (comparing 2 or more groups) or independent student's t test (comparing between 2 groups only). Significance = p value < 0.05 * $P < 0.01$ ** $P < 0.001$ ***, $P<0.0001$ ****.

6.3 Results

6.3.1 Optimising TR146 MUC1 Cell Model Using UWMS

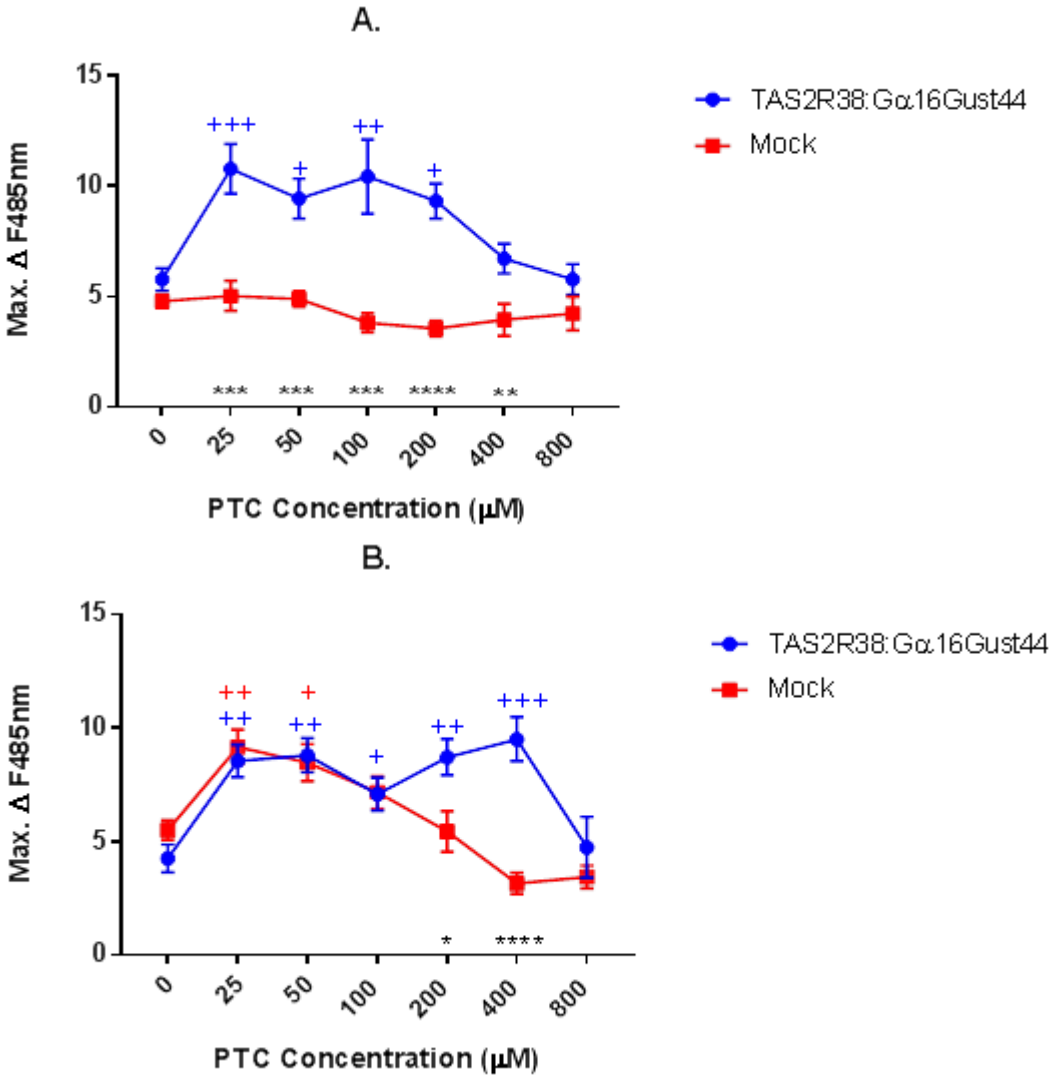

Figure 6-1 FLEX station measurement of intracellular calcium with PTC in TR146 MUC1 cells with and without UWMS

TR146 MUC1 Cells were loaded with the fluo-4am calcium indicator and incubated with (B.) or without (A.) UWMS. Fluorescence emissions were recorded using FLEX station fluorescence plate reader, before and after addition of increasing concentrations of PTC. The panels show mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition, in mock transfected and TAS2R38:Ga16Gust44 co-transfected TR146 MUC1 cells. Data is representative of 2-3 experiments (4-6 analyses). Analysed for statistical significance using one-way ANOVA with Tukey's multiple comparisons (difference from baseline = +) and independent student's T test (difference between mock and TAS2R38 transfected cell lines = *). Significance = p value < 0.05 */+ P < 0.01 **/++ P < 0.001 ***/+++, P<0.0001 ****/++++.

To prepare the cell models for use with older and younger saliva samples, control UWMS was used and cells were stimulated with bitter compounds following incubation with saliva. As a single donor saliva was used, this experiment only represents preliminary work to optimise the model for use with saliva and is not representative of the responses in the presence of saliva in general. In line with previous observations, TAS2R38:Gα16Gust44 transfected TR146 cells responded, with elevated intracellular calcium, to PTC in a dose dependent manner (Figure 6-1 A). The response to 25, 50 100 and 200μM PTC was significantly higher than the response to saline alone in TAS2R38:Gα16Gust44 transfected cells, with mean (+/-SEM) peak fluorescence of 10.78 +/- 1.12 (p=<0.0001), 9.434 +/- 0.90 (p=<0.05), 10.43 +/- 1.676 (p=<0.01) and 9.326 +/- 0.79 (p=<0.05) respectively, compared to 5.78 +/- 0.50 (arbitrary values). In mock transfected cells, there was no significant increase in intracellular calcium following PTC stimulation (Figure 6-1 A). TAS2R38:Gα16Gust44 transfected cells displayed significantly higher responses to all concentrations of PTC, apart from 800μM, compared to mock transfected cells with an average response of 8.32 +/- 0.91, compared to 4.33 +/- 0.52. In mock transfected cells, the addition of UWMS led to a significantly increased calcium response to 25, 50, 100 and 200μM PTC, with values of 9.16 +/- 0.77 (p=<0.001), 8.47 +/- 0.82 (p=<0.001), 7.17 +/- 0.72 (p=<0.001) and 5.45 +/- 0.89 (p=<0.05) respectively, compared to values of 5.03 +/- 0.68, 4.90 +/- 0.35, 7.7 +/- 3.81 and 5.45 +/- 3.19 without WMS (Figure 6-1 B). In TAS2R38:Gα16Gust44 transfected cells, there was no significant difference in response to PTC with or without WMS except for 400μM (Figure 6-1 B). At this concentration, the response was significantly higher in the presence of WMS, 9.50 +/- 0.97, compared to 6.71 +/- 0.67, without saliva.

6.3.2 Optimising SCC090 Cell Model Using UWMS

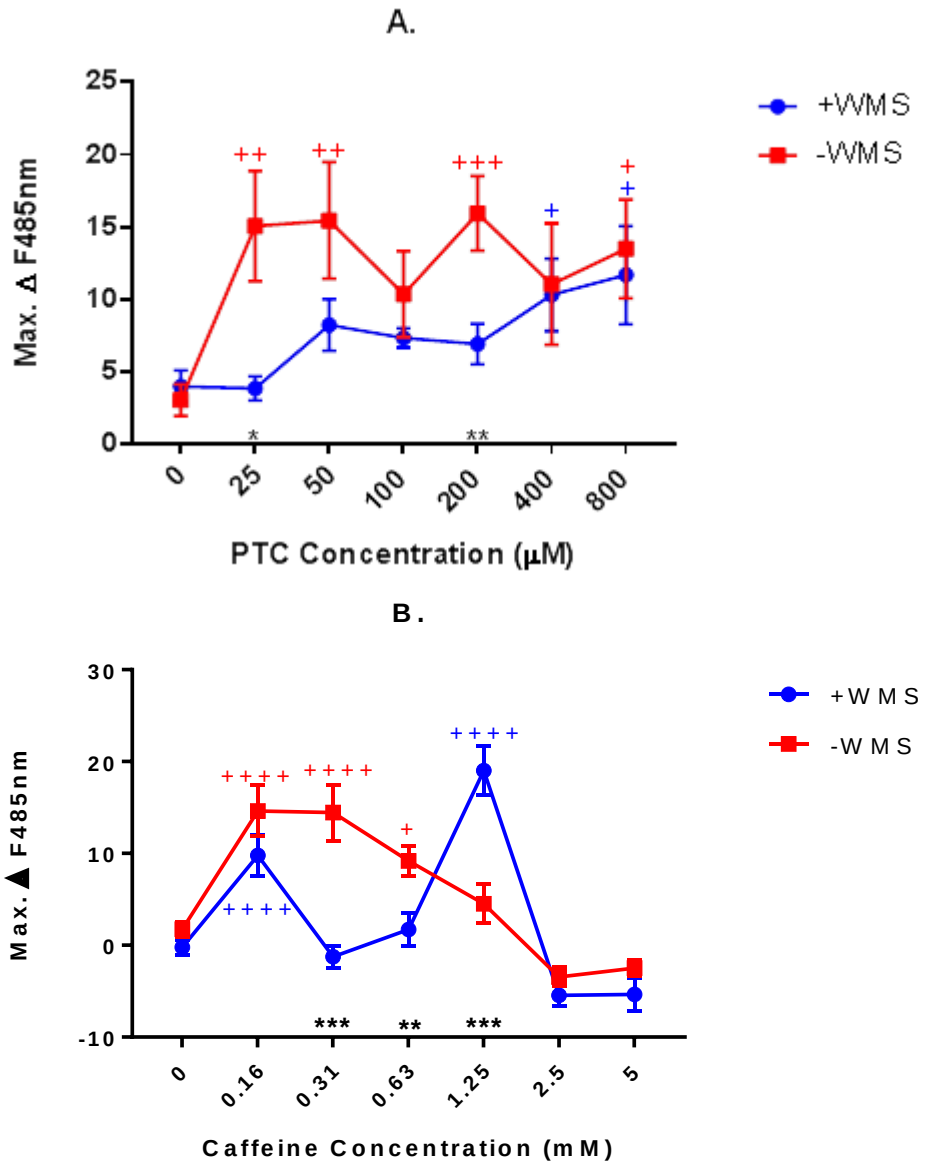

Figure 6-2 FLEX station measurement of intracellular calcium with PTC and Caffeine in SCC090 cells with and without UWMS

SCC090 cells were loaded with the fluo-4am calcium indicator and incubated with or without UWMS. Fluorescence emissions were recorded using FLEX station fluorescence plate reader, before and after addition of increasing concentrations bitter compounds. The panels show mean (+/- SEM) change (Δ) in fluorescence from baseline addition of compound, PTC (TAS2R38 agonist, A.) and caffeine (TAS2R10 agonist, B.). Data is representative of 2-3 experiments (4-6 analyses). Analysed for statistical significance using Kruskal-Wallis test with Dunn's multiple comparisons (A.) or one-way ANOVA with Dunnetts's multiple comparisons (B.) (difference from baseline = +) and independent student's T test (difference between -WMS and +WMS = *). Significance = p value < 0.05 */+ P < 0.01 **/++ P < 0.001 ***/+++, P<0.0001 ****/++++.

The effect of UWMS on calcium responses to bitter tastants was also demonstrated in SCC090 cells, which were shown in chapter 5 to express TAS2R receptors endogenously. As expected, SCC090 cells responded to both PTC and caffeine in a dose dependent manner. PTC concentrations of 25, 50, 200 and 800µM elicited significantly greater mean (+/-SEM) fluorescence responses of 15.07 +/- 3.82 (p=<0.01), 15.45 +/- 4.03 (p=<0.01), 15.95 +/- 2.58 (p=<0.001) and 13.5 +/- 3.41 (p=<0.05) respectively, compared to the response to carrier control which gave a fluorescence response of 3.05 +/- 1.09 (Figure 6-2 A). Addition of UWMS led to significantly reduced responses to 25 and 200µM PTC, with respective fluorescence responses of 3.86 +/- 0.83 (p=<0.05) and 6.93 +/-1.4 (p=<0.01). For caffeine, responses, 0.16, 0.31 and 0.63mM elicited significantly greater responses of 14.62 +/- 2.78 (p=<0.0001), 14.44 +/-3.04 (p=<0.0001) and 9.18 +/- 1.64 (p=<0.05) respectively, compared to a response of 1.76 +/- 0.83 for the carrier control (Figure 6-2 B). In the presence of UWMS, significantly reduced calcium responses were seen for 0.31 and 0.63mM caffeine with fluorescence values of -1.259 +/- 1.20 (p=<0.001) and 1.71 +/- 1.78 (p=<0.01) respectively. Conversely, at 1.25mM caffeine, responses were significantly greater in the presence of UWMS with fluorescence of 19.00 +/- 2.68 (p=<0.001) compared to 4.52 +/- 2.14 without UWMS.

6.3.3 Interaction Between PTC and TAS2R38 in TR146 MUC1 Cells with UWMS from Older and Younger Adults

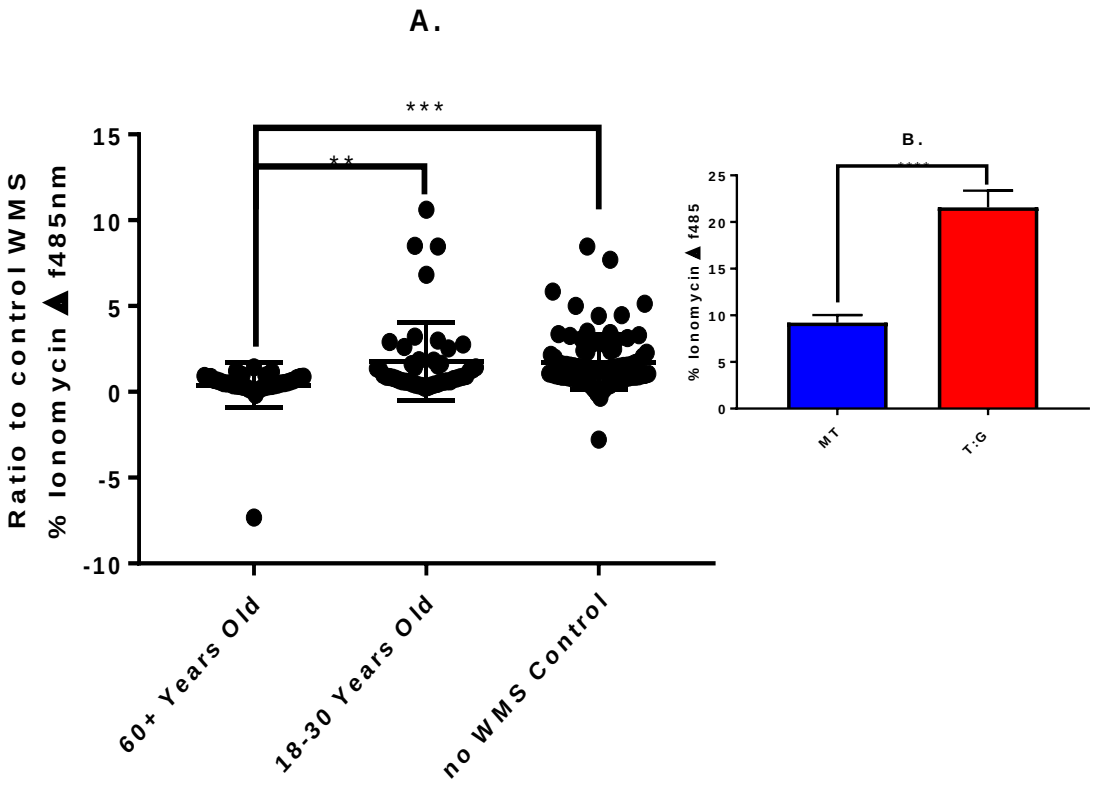

Figure 6-3 FLEX station measurement of intracellular calcium with PTC in TR146 MUC1 cells with and without UWMS from older and younger adults

TR146 MUC1 Cells were loaded with the fluo-4am calcium indicator and incubated with or without UWMS from older (60+ years, n=22) and younger (18-30 years, n=29) adults. Fluorescence emissions were recorded using FLEX station fluorescence plate reader, before and after addition of 25µM PTC. A. Mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition (normalised to ionomycin response and control WMS response), in TAS2R38:Ga16Gust44 co-transfected TR146 MUC1 cells. Cells stimulated with 25µM PTC without WMS used for comparison (n=44). Data is representative of 2 experiments. B. Transfection efficiency of TR146 MUC1 cells, without WMS. Mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition, in mock transfected (MT) and TAS2R38:Ga16Gust44 co-transfected (T:G) TR146 MUC1 cells (n=20). Analysed for statistical significance using independent student's T test. Significance = p value < 0.05 * P < 0.01 ** P < 0.001 ***.

The effect of age-related changes in UWMS on calcium responses to PTC were demonstrated by incubating TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells with UWMS from older and younger adults. 25μM concentration of PTC was selected for the experiment since it was the lowest concentration required to elicit a TAS2R38 specific response, i.e. a significantly greater response in TAS2R38:Gα16Gust44 transfected cells compared to mock transfected. A significantly reduced response to PTC was shown in the presence of UWMS from older adults (n=22) with a mean (+/- SEM) fluorescence value of 0.39 +/- 0.21 compared to 1.76 +/- 0.33 with UWMS from younger adults (n=29) and 1.71 +/- 0.17 ($p=<0.01$) without UWMS ($p=<0.001$) (arbitrary values) (Figure 6-3 A). There was no significant difference between responses with younger UWMS and responses without UWMS. Successful transfection was demonstrated since the response to PTC was significantly greater in TAS2R38:Gα16Gust44 transfected cells, 21.57 +/- 0.81, compared to the response in mock transfected cells which was 9.22 +/-1.80 ($p=<0.0001$) (Figure 6-3 B).

Table 6-1 Kendall's tau b correlation between calcium response to PTC in TR146 MUC1 cells and taste perceptions in older and younger adults.

Correlations				
	Age of Participant			Response to PTC in TR146 MUC1
Kendall's tau_b	18-30 Years Old	TP water rinse 1-10	Correlation Coefficient	-.209
			Sig. (2-tailed)	.104
		TP menthol odour 1-10	Correlation Coefficient	.390**
			Sig. (2-tailed)	.004
		TP menthol 1-10	Correlation Coefficient	.265
			Sig. (2-tailed)	.050
		TP caffeine 1-10	Correlation Coefficient	.364**
			Sig. (2-tailed)	.007
		TP MSG 1-10	Correlation Coefficient	.007
			Sig. (2-tailed)	.957
		TP capsaicin 1-10	Correlation Coefficient	-.063
			Sig. (2-tailed)	.639
	60+ Years Old	TP water rinse 1-10	Correlation Coefficient	-.196
			Sig. (2-tailed)	.180
		TP menthol odour 1-10	Correlation Coefficient	.053
			Sig. (2-tailed)	.737
		TP menthol 1-10	Correlation Coefficient	-.148
			Sig. (2-tailed)	.334
		TP caffeine 1-10	Correlation Coefficient	.058
			Sig. (2-tailed)	.706
		TP MSG 1-10	Correlation Coefficient	-.026
			Sig. (2-tailed)	.862
		TP capsaicin 1-10	Correlation Coefficient	-.108
			Sig. (2-tailed)	.479
**. Correlation is significant at the 0.01 level (2-tailed).				
*. Correlation is significant at the 0.05 level (2-tailed).				

Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of 25µM PTC, in presence of saliva from older and younger adults. Average perceived intensity of taste and TRP agonists by subjective measurement on a scale of 0-10 (data presented in chapter 3). TP=taste/TRP perception. Older adults, 60+ years, n=22. Younger adults, 18-30 years, n=29.

Correlation analysis was performed between calcium responses to PTC, in TR146 MUC1 cells and perceived intensity of taste and TRP stimulants, measured in chapter 3. In younger adults, there was a significant positive correlation between the PTC response, in the presence of UWMS, and taste perception of caffeine ($p=<0.01$) (Table 6-1). However, a similar correlation was shown for menthol odour in younger adults ($p=<0.01$) which would not be affected by saliva. There were no correlations between TR146 MUC1 calcium response to PTC, in the presence of UWMS from older adults, with taste perceptions of that age group however it should be noted that only a small subset of subjects were tested for PTC perception which may have precluded significant correlations.

Table 6-2 Kendall's tau b correlation between calcium response to PTC in TR146 MUC1 cells and salivary total protein, carbonic anhydrase 6 and cystatin S, in older and younger adults.

Correlations			
			Response to PTC in TR146 MUC1
Kendall's tau_b	Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	-.017
		Sig. (2-tailed)	.852
	Cystatin S in resting saliva	Correlation Coefficient	-.213*
		Sig. (2-tailed)	.023
	Total Protein Resting Saliva	Correlation Coefficient	.051
		Sig. (2-tailed)	.586
**. Correlation is significant at the 0.01 level (2-tailed).			
*. Correlation is significant at the 0.05 level (2-tailed).			

Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of 25µM PTC, in presence of saliva from older and younger adults. Carbonic anhydrase 6 and cystatin S measured using immuno-blot of UWMS. Total protein quantified using BCA assay (data presented in chapter 3). Older adults, 60+ years, n=22. Younger adults, 18-30 years, n=29.

Salivary cystatin S levels could be negatively correlated with the PTC response in TR146 MUC1 cells, in the presence of UWMS from both age groups (p=<0.05) (Table 6-2).

Table 6-3 Kendall's tau b correlation between calcium response to PTC in TR146 MUC1 cells and salivary mucin in older and younger adults.

Correlations				
	Age of Participant			Response to PTC in TR146 MUC1
Kendall's tau_b	18-30 Years Old	MUC5B PAS	Correlation Coefficient	.218
			Sig. (2-tailed)	.090
		MUC7 PAS	Correlation Coefficient	.283*
			Sig. (2-tailed)	.028
		SNA	Correlation Coefficient	.099
			Sig. (2-tailed)	.443
		MALII	Correlation Coefficient	.223
			Sig. (2-tailed)	.084
	60+ Years Old	MUC5B PAS	Correlation Coefficient	-.261
			Sig. (2-tailed)	.074
		MUC7 PAS	Correlation Coefficient	-.123
			Sig. (2-tailed)	.399
		SNA	Correlation Coefficient	-.167
			Sig. (2-tailed)	.254
		MALII	Correlation Coefficient	-.145
			Sig. (2-tailed)	.321
**. Correlation is significant at the 0.01 level (2-tailed).				
*. Correlation is significant at the 0.05 level (2-tailed).				

Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of 25µM PTC, in presence of saliva from older and younger adults. Salivary mucin measured using SDS-PAGE gel electrophoresis with PAS staining of glycoproteins and immuno-blotting for MUC5B, MUC7 and lectins, SNA and MALII (data presented in chapter 3 and 4). Older adults, 60+ years, n=22. Younger adults, 18-30 years, n=29.

To determine whether salivary mucin levels and sialylation could be linked with the resulting UWMS PTC responses observed in TR146 MUC1 cells, correlation analysis was also conducted for MUC5B, MUC7 and sialic acid levels in UWMS of older and younger adults. Only MUC7 from UWMS of younger adults could be positively correlated with the PTC response in TR146 MUC1 cells, in the presence of saliva from that age group ($p=<0.05$) (Table 6-3).

6.3.4 Calcium Responses to Caffeine in SCC090 Cells with UWMS from Older and Younger Adults

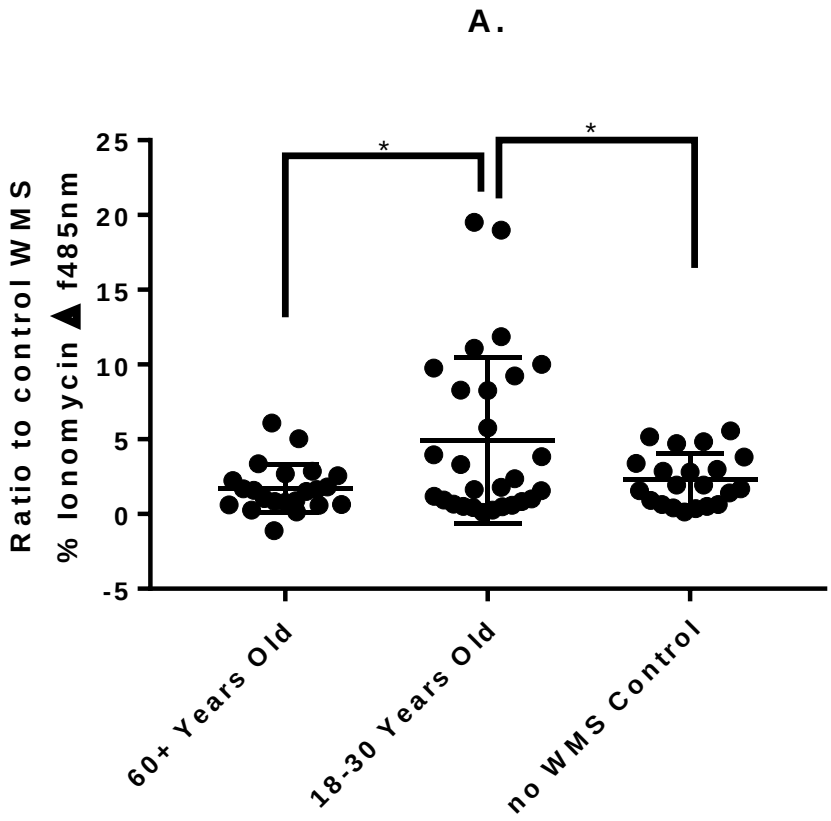

Figure 6-4 FLEX station measurement of intracellular calcium with caffeine in SCC090 cells with and without UWMS from older and younger adults

SCC090 cells were loaded with the fluo-4am calcium indicator and incubated with or without UWMS from older (60+ years, n=22) and younger (18-30 years, n=28) adults. Fluorescence emissions were recorded using FLEX station fluorescence plate reader, before and after addition of 0.16mM caffeine. Mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition (normalised to ionomycin response and control WMS response). Cells stimulated with 0.16mM caffeine without WMS used for comparison (n=21). Analysed for statistical significance using independent student's T test. Significance = p value < 0.05 * P < 0.01 ** P < 0.001 ***.

To demonstrate the effect of age-related changes in UWMS on taste receptor interactions with caffeine, intracellular calcium responses in SCC090 cells were measured in the presence of UWMS from older and younger adults. A significantly higher response to 1.6mM caffeine was seen in the presence of UWMS from younger adults, with a mean fluorescence value (+/-SEM) of 4.94 +/- 1.05, compared to with UWMS from older adults, 1.72 +/-0.35 ($p=<0.05$) and without saliva, 2.3 +/- 0.38 ($p=<0.05$) (arbitrary values) (Figure 6-4). The response in the presence of UWMS from older adults was not significantly different to the response seen without UWMS.

Table 6-4 Kendall's tau b correlation between calcium response to caffeine in SCC090 cells and taste perceptions in older and younger adults.

	Age of Participant			Response to caffeine SCC090
Kendall's tau_b	18-30 Years Old	TP water rinse 1-10	Correlation Coefficient	-.163
			Sig. (2-tailed)	.205
		TP menthol odour 1-10	Correlation Coefficient	.097
			Sig. (2-tailed)	.478
		TP menthol 1-10	Correlation Coefficient	.083
			Sig. (2-tailed)	.538
		TP caffeine 1-10	Correlation Coefficient	.268*
			Sig. (2-tailed)	.046
		TP MSG 1-10	Correlation Coefficient	.051
			Sig. (2-tailed)	.704
		TP capsaicin 1-10	Correlation Coefficient	.063
			Sig. (2-tailed)	.639
	60+ Years Old	TP water rinse 1-10	Correlation Coefficient	.043
			Sig. (2-tailed)	.771
		TP menthol odour 1-10	Correlation Coefficient	.026
			Sig. (2-tailed)	.869
		TP menthol 1-10	Correlation Coefficient	.047
			Sig. (2-tailed)	.765
		TP caffeine 1-10	Correlation Coefficient	-.067
			Sig. (2-tailed)	.668
		TP MSG 1-10	Correlation Coefficient	-.020
			Sig. (2-tailed)	.894
		TP capsaicin 1-10	Correlation Coefficient	.055
			Sig. (2-tailed)	.726
		TP menthol 1-10	Correlation Coefficient	.047
			Sig. (2-tailed)	.765
**. Correlation is significant at the 0.01 level (2-tailed).				
*. Correlation is significant at the 0.05 level (2-tailed).				

Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of caffeine, in presence of saliva from older and younger adults. Average perceived intensity of taste and TRP agonists by subjective measurement on a scale of 0-10 (data presented in chapter 3). TP=Taste/TRP Perception. Older adults, 60+ years, n=22. Younger adults, 18-30 years, n=28.

As with PTC responses, a correlation analysis was performed between calcium responses to caffeine, in SCC090 cells and perceived intensity of taste and TRP stimulants, measured in chapter 3. The perceived intensity of caffeine, in younger adults, could be positively correlated with the calcium response in SCC090 cells in the presence of saliva from younger adults ($p=<0.05$) (Table 6-4). There were no other significant correlations between the response to caffeine in SCC090 cells, in the presence of UWMS and taste perceptions in either age group.

Table 6-5 Kendall's tau b correlation between calcium response to caffeine in SCC090 cells and salivary total protein, carbonic anhydrase 6 and cystatin S, in older and younger adults.

Correlations		
		Response to caffeine in SCC090 cells
Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	.026
	Sig. (2-tailed)	.782
Cystatin S in resting saliva	Correlation Coefficient	.254**
	Sig. (2-tailed)	.007
Total Protein Resting Saliva	Correlation Coefficient	-.016
	Sig. (2-tailed)	.866
**. Correlation is significant at the 0.01 level (2-tailed).		
*. Correlation is significant at the 0.05 level (2-tailed).		

Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of caffeine, in presence of saliva from older and younger adults. Carbonic anhydrase 6 and cystatin S measured using immuno-blot of UWMS. Total protein quantified using BCA assay (data presented in chapter 3). Older adults, 60+ years, n=22. Younger adults, 18-30 years, n=29.

To see the effect of salivary protein composition on the resulting response to caffeine in SCC090 cells, with UWMS, a correlation was performed with cystatin S, carbonic anhydrase and total protein levels measured in chapter 3. Cystatin S levels in UWMS from adults of all ages, was positively correlated with the caffeine-induced calcium response measured in SCC090 cells in the presence of UWMS ($p=<0.01$) (Table 6-5). Total salivary protein and carbonic anhydrase 6 could not be correlated with caffeine responses.

Table 6-6 Kendall's tau b correlation between calcium response to caffeine in SCC090 cells and salivary mucin in older and younger adults.

****. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).**				
	Age of Participant			Response to caffeine in SCC090 cells
Kendall's tau_b	18-30 Years Old	MUC5B PAS	Correlation Coefficient	.159
			Sig. (2-tailed)	.218
		MUC7 PAS	Correlation Coefficient	.168
			Sig. (2-tailed)	.193
		SNA	Correlation Coefficient	-.053
			Sig. (2-tailed)	.682
		MALII	Correlation Coefficient	.007
			Sig. (2-tailed)	.957
		MUC5B blot	Correlation Coefficient	-.356**
			Sig. (2-tailed)	.006
		MUC7 blot	Correlation Coefficient	-.039
			Sig. (2-tailed)	.762
	60+ Years Old	MUC5B PAS	Correlation Coefficient	-.360*
			Sig. (2-tailed)	.016
		MUC7 PAS	Correlation Coefficient	-.004
			Sig. (2-tailed)	.979
		SNA	Correlation Coefficient	-.170
			Sig. (2-tailed)	.256
		MALII	Correlation Coefficient	-.233
			Sig. (2-tailed)	.119
		MUC5B blot	Correlation Coefficient	-.241
			Sig. (2-tailed)	.107
		MUC7 blot	Correlation Coefficient	.012
			Sig. (2-tailed)	.937

Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of caffeine, in presence of saliva from older and younger adults. Salivary mucin measured using SDS-PAGE gel electrophoresis with PAS staining of glycoproteins and immuno-blotting for MUC5B, MUC7 and lectins, SNA and MALII (data presented in chapter 3 and 4). Older adults, 60+ years, n=22. Younger adults, 18-30 years, n=29.

The correlation analysis was repeated for mucin levels and sialylation in UWMS and caffeine responses in SCC090 cells, in the presence of UWMS from older and younger adults. In both age groups, MUC5B levels was negatively correlated with caffeine response in SCC090 cells in the presence of saliva from younger and older adults ($p=<0.01$ and $p=<0.05$ respectively) (Table 6-6).

6.4 Discussion

In vitro models for taste receptor activation are widely used to study the interaction between taste compounds and receptors (Brockhoff et al. 2007, Bufe et al. 2002, Tsien et al. 1985, Meyerhof et al. 2010). However, previous studies have used cell lines from extra oral locations which may not be representative of the tongue epithelium. Moreover, the role played by saliva, in tastant-receptor interaction has not been considered. In this study, a biologically relevant model for taste receptor activation was developed, using oral epithelial cells. Results from chapter 5 demonstrated that the cell model could successfully be used to measure calcium responses to bitter taste compounds *in vitro*. To study the effect of saliva on calcium responses from TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells and on endogenous TAS2R responses from SCC090 cells, the cells were incubated with saliva and taste compounds were applied on top of the saliva layer. Use of a single donor UWMS gave insight into the effects of saliva on the cell models. In TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells, the addition of UWMS did not significantly alter calcium responses to PTC except for responses to 400μM PTC which were increased. This implies that saliva may increase TAS2R38 independent responses to PTC, since concentrations above 100μM likely recruit other response pathways. Previous studies have shown that at least two, separate, intracellular calcium response pathways, occur in response to bitter taste compounds (Hacker et al. 2008). In cells which do not express TAS2Rs, calcium responses to high concentration of bitter agonists have been shown (Meyerhof et al. 2010). This indicates TAS2R independent mechanisms for calcium responses to bitter ligands, which may be enhanced by saliva. In mock transfected cells, none of the PTC concentrations tested influenced intracellular calcium response without WMS. Therefore, WMS was required for the compound to elicit a response. This further supports the idea that saliva may increase TAS2R38 independent responses to PTC. It may be that, in the presence of saliva, lower concentrations of PTC are required to activate TAS2R38 independent response pathways due to enhanced solubilisation of the compound. In line with this theory, responses to higher concentrations of PTC, which likely stimulate TAS2R38 independent pathways, were also slightly increased in SCC090 cells.

When there was no salivary layer, TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells responded to PTC in a dose dependent manner. Intracellular calcium responses

began to decrease at concentrations higher than 100µM. As previously discussed in chapter 5, this may be due to a toxic effect of high concentrations of PTC or due to incomplete solubilisation of the compound at high concentrations. Saliva appeared to negate this effect, as the response to 400µM PTC was significantly greater than the response to the carrier control. There are two proposed mechanisms for this. Firstly, saliva may have a protective effect which prevents the toxicity of PTC. Previous studies have demonstrated the protective effects of saliva on the oral epithelium, with reduced penetration of florescent dyes into the cells of the oral mucosa, in the presence of saliva (Adams 1974, Wallenius 1966). Reduced penetration of PTC may mean that cells are exposed to lower levels, and thus reduced toxicity. However, toxicity of PTC is unlikely since previous studies have shown high tolerance for PTC, in mammalian cells, at much greater concentrations than those used in this study (Elia et al. 1994). Alternatively, saliva may enhance the solubility of PTC and therefore improve its availability to bind with the receptor. Saliva may solubilise compounds which are otherwise water insoluble because of the presence of proteins which have a detergent-like effect on lipophilic compounds (Hutteau and Mathlouthi 1998, Ginsburg et al. 2012). WMS and mucin have been shown to improve solubility of lipophilic plant polyphenols (Ginsburg et al. 2012).

As discussed in chapter 5, GPCRs, including TAS2Rs (Robinett et al. 2011, Meyerhof et al. 2005, Bufe et al. 2002), may be internalised and then down-regulated upon agonist stimulation (Koenig and Edwardson 1997, Gray and Roth 2001, Kallal et al. 1998). The internalisation of GPCRs occurs rapidly as a de-sensitisation mechanism (Lefkowitz 1998). Internalisation of GPCRs results in abolished availability to participate in signal transduction and likely represents the preceding step before the receptor is either recycled or degraded, resulting in down-regulation (Li, Benovic and Liu-Chen 2000). Rapid desensitization occurs following ligand induced phosphorylation of the receptor, followed by interaction of the receptor with arrestin proteins in the cell cytoplasm, which prevent coupling between the receptor and G protein (Lefkowitz et al. 1997). Internalised receptors are shuttled toward intracellular membranes to be de-phosphorylated by endosome-associated phosphatases (Pitcher et al. 1995). Dephosphorylated GPCRs are then recycled back to the to the plasma membrane, allowing for recovery of cell signalling potential (re-sensitisation) (Pitcher et al. 1995). Some internalised GPCRs undergo endocytosis, following agonist stimulation, via clathrin-coated pits, and are trafficked to the lysosomes where they are subject to

proteolysis (Tsao, Cao and von Zastrow 2001). Increased availability of PTC, to the receptor, in the presence of saliva, may therefore enhance this mechanism. This could explain why there was a slight reduction in responses in TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells and SCC090 cells in the presence of saliva. Meanwhile, TAS2R38 independent responses would not be affected by this and as such, the responses in mock-transfected cells were increased in the presence of saliva. This corroborates previous reports of TAS2R desensitisation, since stimulation with higher concentrations of potent TAS2R1 agonists, was shown to cause reduced intracellular calcium responses in transiently transfected C6-glioma cells (Upadhyaya et al. 2010).

It is important to note however, that the time periods investigated in this study may not be sufficient for TAS2R down-regulation to occur. While receptor internalisation occurs in seconds or minutes, down-regulation and reduction of receptor numbers occurs over hours or days following repeated ligand stimulation (Tsao et al. 2001, Ko et al. 1999). In this study, cells were exposed to ligands for a period of less than 2 minutes, which may have been sufficient to induce internalisation of receptors, but it is unlikely that down-regulation would occur.

SCC090 cells were shown, in chapter 5, to express several taste receptors for caffeine endogenously and were therefore used to model the effect of saliva on caffeine responses. UWMS reduced responses to lower concentrations of caffeine, while at higher concentrations there was an increase in calcium response in the presence of saliva. Caffeine has previously been shown to cause reduced cell viability in a dose dependent manner (Rayburn et al. 2001, Fernandez et al. 2003). Saliva may provide some protection against this and therefore responses to 1.25mM caffeine were increased in the presence of saliva. It is possible that the availability of caffeine molecules was also enhanced by saliva. Like PTC, caffeine is sparingly water soluble, and may interact with salivary proteins. Ligand induced internalisation or down-regulation may also apply to caffeine TAS2R receptor expression. As such, responses to lower concentrations, which may activate TAS2Rs, were slightly reduced by WMS.

It is also possible that saliva, from certain individuals, acts as a barrier to taste compounds, preventing their access to the receptors. However, if that were the case, it is not clear why the response in mock transfected TR146 MUC1 cells would be enhanced by the presence of saliva. However, enhanced responses to higher concentrations of caffeine and PTC in SCC090 and TAS2R38:Gα16Gust44 transfected TR146 MUC1

cells, respectively, may be fitting with a saliva-barrier theory since higher concentrations would be needed to elicit a response. However, if this were the case then it is not clear why there were robust responses to the lowest concentration of caffeine in SCC090 cells in the presence of WMS. It is important to note, however, that initial optimisation experiments were conducted with UWMS from one individual therefore, due to wide variation in composition and physical properties of saliva between individuals, this may not be representative of the effect of saliva in general.

This model demonstrates the complexity of bitter taste response pathways since there is not always a positive response-concentration correlation. *In vivo* studies have shown that perceived intensity does not correlate to concentration, for some bitter compounds (Mojet et al. 2005, Webb et al. 2015). Further, the pattern of responses is dependent on compound, as different compounds may recruit different response mechanisms. For example, some bitter taste compounds, including caffeine, can diffuse across the cell membrane to activate G-proteins directly (Peri et al. 2000, Naim et al. 1994). Furthermore, some compounds such as quinine and caffeine, can inhibit down-stream taste receptor signalling components and therefore are perceived less intensely at higher concentrations (Talavera et al. 2008, Gees et al. 2014). It likely therefore, that there are multiple pathways for intracellular calcium responses caused by bitter ligands, and that saliva has a differential effect on each pathway.

A putative mechanism for TAS2R independent responses to PTC in TR146 cells was suggested in chapter 5, as GPR20, an orphan GPCR, was up-regulated by PTC stimulation and this coincided with a down-regulation of melatonin receptor 1. These may form a heterodimeric receptor which could be responsible for the raised intracellular calcium seen in response to high concentrations of PTC in SCC090 and TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells.

Other ion channels may also be involved in non-specific calcium responses to PTC. Previous studies have shown that voltage gated calcium channels (VGCCs) may be activated by bitter taste compounds, leading to an increase in intracellular calcium in some type ii taste cells (Hacker et al. 2008). In the intestinal cell line, STC-1, L-type voltage-sensitive calcium channels have been shown to play a role in calcium responses to bitter agonists (Chen et al. 2006). Some bitter taste compounds activate ryanodine receptor type 1 (RyR1) channels expressed by the endoplasmic reticulum (Rebello and Medler 2010). RyR1 channels are opened in a calcium-induced calcium-release

mechanism and have been shown to contribute to calcium responses to bitter compounds in a sub-set of taste cells (Rebello and Medler 2010). RyR1 channels preferentially associate with calcium influx from VGCCs and are less affected by calcium release from IP3R3, due to direct coupling between Ryears and VGCCs (Rios and Brum 1987, Rebello and Medler 2010).

6.4.1 Effect of Saliva from Older and Younger Adults on Calcium Responses to PTC – *In vitro*

To see whether age-related changes in UWMS physical properties and mucin composition might affect tastant-receptor interactions, saliva samples from older and younger adults were analysed using the cell models. Calcium responses to PTC in TAS2R38 transfected TR146 cells were significantly reduced in the presence of UWMS from older adults, compared with responses in buffer alone. There was no significant difference in PTC responses with UWMS from younger adults, compared to without saliva. This suggests that, with saliva from older adults, transport of PTC to the TAS2R38 receptor may be reduced.

Age-related changes in saliva, which were demonstrated in chapters 3 and 4, may be responsible for this effect. Reduced mucin levels and changes in sialylation could reduce hydrophobic interactions between PTC and saliva. Additionally, UWMS from older adults had reduced muco-adhesion and reduced viscoelasticity which could affect dissolution of taste compounds. However, there were no significant correlations between viscoelasticity and muco-adhesive capacity of UWMS with PTC calcium responses observed in TR146 MUC1 cells, in the presence of saliva from either age group (data shown in Appendix 20).

Analyses indicated a negative correlation between salivary levels of cystatin S and PTC responses in TR146 cells, with UWMS. Previous studies have also shown a link between increased cystatin S in saliva and reduced taste perception for caffeine/urea (Dsamou et al. 2012, Morzel et al. 2014). This has been suggested to be caused by the inhibitory action against cysteine proteases - mediated by cystatin S, which may lead to a more viscous saliva and a barrier to taste receptors (Dsamou et al. 2012). However, as shown in chapter 4 of this thesis, UWMS from older adults was not significantly more viscous than UWMS of younger adults and as such, it seems unlikely that older saliva acts as a physical barrier to taste receptors. Furthermore, viscosity of UWMS was not

significantly correlated with PTC responses in TR146 MUC1 cells (data shown in Appendix 20) and in previous chapters, could not be correlated with UWMS cystatin S levels either. It is not clear therefore, why salivary cystatin S might be linked to reduced PTC-TAS2R38 activation.

MUC7 levels in UWMS from younger adults was positively correlated with the calcium response in the presence of UWMS from that age group. Therefore, higher levels of salivary MUC7 may result in enhanced TAS2R38 activation. This provides insight into the mechanisms behind age-related reduction in taste perception of PTC. In chapter 1 and 5 of this study, perceived intensities of PTC, as well as calcium responses to PTC in the cell model, were significantly reduced in older adults compared to younger and, in chapter 4, salivary MUC7 was also shown to be reduced, in this age group. Furthermore, other studies have found that taste thresholds for lipophilic bitter compounds are more affected by age-related decline in taste sensation compared to less lipophilic compounds (Schiffman et al. 1994a). *In vivo*, hydrophobic interactions between tastant molecules and the hydrophobic regions of the peptide backbone of salivary mucin influence diffusion through the mucosal pellicle (Larhed et al., 1997). Taste compounds with greater lipophilicity and hydrophobicity have increased interaction with mucin (Larhed et al., 1997; Matthes et al., 1992; Norris and Sinko, 1997). Salivary mucins closely associate with the oral mucosa via mucin-mucin interactions with surface bound MUC1, therefore taste compounds bound in this network may have greater availability to receptors expressed by epithelial cells. Previous studies have shown that saliva and mucin can increase availability of lipophilic polyphenols as well as enhancing retention in the oral cavity (Ginsburg et al. 2012).

In addition, thiol groups present in PTC may increase its interaction with salivary mucin. Thiolation of drug compounds leads to formation of disulphide bonds between sulfhydryl groups and cysteine rich domains of mucin via thiol-disulphide exchange reactions (Bernkop-Schnürch, Schwarz and Steininger 1999). This has been shown to increase muco-adhesion of thiol- compounds, with increased retention at the mucosal surface, enhancing diffusion through the mucus layer because of proximity of the compound to the receptor membrane (Bernkop-Schnürch, Guggi and Pinter 2004, Dünnhaupt et al. 2011, Clausen and Bernkop- Schnürch 2000). Reduced salivary MUC7 levels in older adults, may therefore result in altered interaction between lipophilic compounds - especially those with a thiol group such as PTC, and salivary

mucin. This could be leading to reduced diffusion to taste receptors and a subsequent reduction in taste sensitivity.

There was a significant positive correlation between caffeine taste perception, in younger adults, and calcium responses to both PTC and caffeine in the cell models, using saliva from this age group. This suggests that saliva from individuals who were more sensitive to caffeine *in vivo*, facilitated increased bitter tastant-receptor interactions *in vitro* – implying a link between saliva and bitter taste function *in vivo*. There were no correlations between taste perception of caffeine in older adults and the responses shown in the cell models, perhaps because of greater heterogeneity of taste perceptions in older adults. However, these results should be interpreted with caution since menthol odour perception in younger adults could also be positively correlated with calcium response to PTC in the cell model. Menthol odour perception would not be affected by saliva and therefore it is not clear why it should correlate with the responses in the cell model. It could be tentatively proposed that the peri-receptor events involved in odour detection are similar to those occurring in taste and that saliva is representative of other biological fluids such as nasal mucus. This would mean that individuals who have saliva which facilitates enhanced taste receptor activation may also have nasal mucus which facilitates enhanced odour perception.

Perception of PTC, assessed in chapter 3, could not be significantly correlated with calcium responses to PTC shown in the cell model. This may be due to differences in the method used for taste testing of PTC and caffeine. PTC was administered as a filter paper strip, which may affect interaction with saliva compared to administration as a solution, since filter paper may adsorb saliva on the tongue. Additionally, only a small subset of volunteers undertook the PTC taste test and therefore it is possible that there were insufficient numbers for conducting correlation analysis.

Salivary proteins have previously been linked with TAS2R38 activation since saliva from PROP tasters has significantly higher levels of two types of basic proline rich proteins (bPRPs), II-2 and Ps-1, compared to saliva from non-tasters (Cabras et al. 2012). Ps-1 may enhance PROP binding to the TAS2R38 receptor by altering the orientation of the molecule, in the TAS2R38 binding pocket (Melis et al. 2013a). Supplementation of saliva with Ps-1, led to enhanced PROP taste perception, even in individuals with the non-taster TAS2R38 phenotype (Melis et al. 2013a). As a related

compound, with similar structure to PROP, PTC taste may also be affected by salivary levels of these proteins.

6.4.2 Effect of Saliva from Older and Younger Adults on Calcium Reponses to Caffeine – *In vitro*

Calcium responses to caffeine were enhanced by the presence of UWMS from younger adults compared to UWMS from older and adults and in cells without UWMS. Saliva may therefore improve dissolution of caffeine or increase availability of the compound to the receptor. Caffeine is amphiphilic in nature, with significant hydrophobic regions which may interact with salivary mucin. These interactions may allow prolonged contact with TAS2Rs and thus perpetuate calcium responses. UWMS from older adults with reduced MUC7 and impaired mucin sialylation could therefore alter binding with caffeine, as proposed for PTC. However, there was no significant correlation between MUC7 levels in UWMS and the calcium response to caffeine in SCC090 cells, in either age group.

There was however, a significant negative correlation between MUC5B levels in UWMS and caffeine induced calcium responses. This suggests that increased MUC5B may lead to reduced caffeine-receptor interaction. MUC5B has been shown to correlate with salivary viscosity (Ligtenberg et al. 2016) while MUC7 is linked with viscoelasticity (Inoue et al. 2008). As such, greater levels of MUC5B may result in a more viscous saliva which could act as a barrier to taste receptors.

Furthermore, UWMS from older adults had less cystatin S compared to UWMS from younger adults. Sweet tasting proteins with structural homology to cystatin S can bind sweet taste receptors and stabilise in the active form, enhancing responses to sweet tastants (Tancredi et al. 2004, Temussi 2011). Monellin is a sweet tasting protein, part of the cystatin family, which can form complexes with a cysteine-rich domain (CRD) of the heterodimeric T1R2–T1R3 receptor, leading to a conformational change in the ligand-binding domain (Temussi 2011). Since heating of monellin, which causes conformational changes, leads to elimination of its binding capacity for T1R2-T1R3, it is apparent that structural homology is of greater importance than sequence, for sweet receptor stabilisation by proteins (Esposito and Temussi 2011). Highly speculatively, it is proposed that cystatin S, which has a similar structure but largely different sequence to monellin (Murzin 1993), could similarly bind to TAS2R receptors and stabilise the

active form. Therefore, it could be tentatively hypothesised that increased cystatin S in UWMS from younger adults could be involved in enhanced receptor activation by caffeine, via stabilisation of the active form of the TAS2R receptor. This could be especially relevant in the case of ligand induced receptor internalisation which may occur in TAS2Rs. Indeed, there was a significant positive correlation between cystatin S levels in UWMS and caffeine responses in SCC090 cells, in the presence of saliva from both age groups. Receptor stabilisation, induced by salivary cystatin S, could therefore reduce ligand induced internalisation, providing a mechanism behind increased calcium responses to caffeine, in SCC090 cells, in the presence of UWMS from younger adults. The link between increased UWMS cystatin S and greater *in vitro* caffeine responses may indicate a receptor specific interaction, since the opposite was shown for PTC responses. The fact that PTC calcium responses were not increased significantly in the presence of UWMS from younger adults, while caffeine responses were, further supports the idea of a receptor/compound specific effect.

The effect of UWMS from younger adults was limited to caffeine response and did not occur with PTC. This could be due to the different structures of caffeine and PTC. PTC is highly lipophilic, with a single hydrophobic aromatic ring and some polar regions (Schiffman et al. 1994a, Biarnés et al. 2010). Caffeine is less lipophilic than PTC but, similarly, has hydrophilic amine and amide groups with a hydrophobic moiety composed of two aromatic rings. Therefore, dissolution in saliva and interactions with salivary proteins may differ between the two compounds. Caffeine is a hydrotrope and can increase the solubility of hydrophobic molecules in water, behaving like a surfactant (Lim and Go 2000, Rasool, Hussain and Dittert 1991). When caffeine interacts with other hydrophobic compounds in this way, its own solubility is improved, and it displays increased diffusivity (Cui 2010). Recent studies have shown that caffeine can also prevent hydrophobic aggregation of certain proteins (Sharma and Paul 2016). Caffeine can encapsulate the protein in clusters, with water molecules, which act as a barrier to physically block interactions between protein molecules, disrupting aggregates (Sharma and Paul 2016). The results from chapter 3 showed that caffeine stimulation resulted in reduced salivary viscoelasticity, in younger adults . It is proposed therefore that caffeine can interact with salivary proteins, possibly mucins, to inhibit their aggregation and thus, allow faster diffusion to reach the taste receptors. In turn, the interaction between caffeine and hydrophobic regions of mucin may enhance the solubility of caffeine, resulting in faster diffusion, as is the case when interactions occur

between caffeine and other hydrophobic compounds (Cui 2010). In older adults, who displayed reduced salivary mucin and reduced sialylation of mucin with associated reduced functionality (i.e. reduced viscoelasticity of saliva), these mechanisms may not operate and as such, the calcium response to caffeine in SCC090 cells, was unaffected by saliva from older adults.

Alternatively, the difference in receptors activated by caffeine and PTC could be a reason for different responses in the presence of UWMS. In this study, it would be reasonable to assume that responses to PTC in TAS2R38 transfected TR146 MUC1 cells occurred via TAS2R38 activation. This is because PTC responses in mock transfected cells were significantly lower than in TAS2R38 transfected cells. However, responses to caffeine in SCC090 cells could originate from several receptors. Expression of TAS2R10 and TAS2R43 were demonstrated in SCC090 cells, in chapter 5 but there are multiple other TAS2Rs, that were not investigated, which could be responsible for caffeine responses. Furthermore, multiple non-taste receptors exist, which may also be activated by caffeine, leading to intracellular calcium release. The lack of specific inhibitors of TAS2R receptors meant it was not possible to clearly demonstrate which receptor is activated during caffeine stimulation of SCC090 cells. Therefore, the effect of saliva on calcium responses to caffeine may not be specific to TAS2Rs. None the less, in TR146 cells, expression of TAS2R10 could not be shown in by RT-PCR, conducted in chapter 5, and only low levels of other TAS2Rs were demonstrated. In line with this, preliminary experiments found no significant calcium responses to caffeine, in TR146 MUC1 cells (Appendix. 27).

Due to the nature of the calcium response assay, the cells were only incubated with saliva for a 20 minute period which may not be sufficient for cell surface mucin binding to occur (Gibbins et al. 2014). Time dependent leakage of the calcium indicator dye was observed in the assay and probenecid could not be used due to its potential inhibitory effect on TAS2R38. Since the layer of saliva was applied after washing out of the fluorophore, the salivary incubation period had to be limited to reduce dye leakage prior to measuring calcium responses. Previous studies have shown that salivary mucin surface binding can be detected after a 1 hour incubation period, but not after 20 minutes (Aroonsang et al. 2014, Gibbins et al. 2014). Therefore, it is unlikely that mucin binding played a significant role in the calcium responses to bitter tastants shown here, in the presence of saliva.

6.5 Concluding Remarks

Overexpression of TAS2R38 in TR146 MUC1 cells, may provide a model for taste receptor activation which is biologically relevant since these cells originate from the oral cavity and express MUC1 to facilitate formation of a mucosal pellicle, as occurs in the oral cavity. Furthermore, SCC090 cells, originating from the human tongue, may also represent a good *in vitro* model of taste function. Saliva can affect the interaction between bitter tastants and their cognate receptors. In TR146 MUC1 and SCC090 cells, a layer of saliva appeared to enhance TAS2R38-independent responses to its ligand, PTC. Calcium responses to caffeine, in SCC090 cells, were also altered by a salivary layer. Saliva from older adults caused reduced PTC-induced TAS2R38 activation, possibly due to changes in salivary mucin composition and rheology which may alter diffusion of PTC. Saliva from younger adults led to enhanced calcium responses to caffeine which did not occur in the presence of saliva from older adults. Caffeine may have improved solubility and diffusivity in saliva compared to in water alone and therefore presence of saliva from younger adults would increase availability of the ligand to the receptor. Meanwhile, in the presence of saliva from older adults, who have reduced salivary mucin sialylation, no such effect was demonstrated. This suggests that tastant-receptor interactions could be reduced in older adults due to impaired quality of saliva, which may subsequently contribute to reduced taste sensation in this age group.

7 General Discussion

In ageing, loss of oral sensory sensitivity can result in malnutrition and diet related diseases, and represents a significant public health risk (Wylie and Nebauer 2011). As such, diet related disorders and malnutrition are highly prevalent in adults aged >60 years old (Prince et al. 2015, Brownie 2006). Saliva is present in the oral cavity and constantly bathes the taste buds on the tongue, where it can interact with sensory stimulants and play a role in oral sensations such as taste, smell and chemo-sensation (Matsuo 2000, Mese and Matsuo 2007). Ageing can lead to reduced salivary flow, particularly from the sub-mandibular/sub-lingual glands (Affoo et al. 2015). However, changes in the physical properties of saliva, such as rheology and composition, including secretion of various salivary proteins, are also evident (Denny et al. 1991, Navazesh et al. 1992, Vissink et al. 1997, Zussman et al. 2007). Previous studies have generally focussed on the effect of ageing on individual salivary elements and have not demonstrated the effect such changes might have on the functionality of saliva. Therefore, this thesis investigated age-related alterations in multiple salivary properties including flow, rheology and protein composition and made links to changes in sensitivity to oral sensations in older adults. A novel *in vitro* model of taste receptor activation was developed, using oral epithelial cell lines, to analyse the effect of saliva from older adults, on taste receptor activation. The overall aim of the thesis was to characterise changes in saliva, which occur in ageing, and how such changes affect oral sensory function in older adults.

7.1 Changes in Sensitivity to Oral Sensations in Older Adults

In chapter 3, it was shown that there was a significant age-related reduction in perception of umami taste and menthol odour. Meanwhile, oral chemo-sensations, elicited by TRP agonists such as capsaicin and menthol, were relatively well retained in older adults. This is in agreement with the literature, since loss of basic taste and odour sensation in older adults has been previously reported, with some differences between the different taste modalities and compound specific effects (Methven et al. 2012). In this study however, there was no age-related difference in caffeine perception which may be due to an inverse concentration-intensity relationship occurring in healthy adults (Keast and Roper 2007). Older adults may not exhibit this phenomenon due to reduced responsiveness to taste compounds in general.

Responsiveness to capsaicin was well retained in older adults, which has also been shown previously, and points to a potential use for some TRP compounds, in improving hedonic aspects of eating for older adults (Fukunaga, Uematsu and Sugimoto 2005a, Forde and Delahunty 2002). However, further research is required to determine acceptability and liking by older adults for trigeminal stimulants in food products. Previous attempts to compensate for taste and smell loss, using TRP agonists, have shown the need for a suitable food system, which is not off putting for older consumers (Koskinen et al. 2003). This is because, preference for novel food flavours may be reduced in older adults, who exhibit a certain degree of food neo-phobia (Bäckström et al. 2003, Tuorila et al. 2001). Also, older adults may have lower preference for chilli compared to younger adults (Guido et al. 2016). Attempts to utilise capsaicin as a compensatory food ingredient should take this into account, perhaps by using just noticeable concentrations, sufficient to increase sensation in a food product, but not too high that the sensation is unpleasant.

In contrast to previous studies, showing that TAS2R38 agonist thresholds are unaffected by age (Abraimov and Mirrakhimov 1979, Whissell-Buechy 1990), perceptions of suprathreshold concentrations of PTC were significantly higher in the younger group in this study. Also, sensitivity to orally administered menthol was not shown to be reduced in older adults, in this study, while others have reported a reduction in menthol perception with age (Kremer et al. 2007b, Koskinen et al. 2003). These discrepancies may be due to differences in sensory testing methods employed and in the concentrations of tastants used. In this study PTC filter paper strips were used while previous studies used solutions. Moreover, the menthol concentration was approximately 6x higher than concentrations tested in previous studies. Also, in the case of menthol sensation, a significant contribution from retro-nasal olfaction is likely to be present and in this study it was shown that menthol odour is significantly reduced in older adults, consistent with previous studies (Murphy 1983). This suggests that higher concentrations of menthol may be required orally, for detection by older adults, compared to younger adults.

The measurements of perceptions of oral sensations are subjective and therefore can be subject to bias (Lawless and Heymann 2010), especially in older adults where cognitive decline may affect the ability to understand rating scales (Methven et al. 2016). Increased saliva secretion, above resting levels, occurs in response to taste, smell and

TRP stimuli (Hector 1999, Nasrawi and Pangborn 1990, Lawless 1984). Therefore, the salivary reflex response to oral stimuli was assessed in this study as a more objective method of measuring sensitivity to oral stimuli. Salivary flow rates were significantly greater in younger adults, to all the tested orally administered compounds except caffeine. A lack of salivary response to caffeine in both age groups supports the idea of reduced responses to supra-threshold concentrations of this compound, as discussed above. Consistent with the reduction in subjective perception, the salivary flow rate response to MSG was reduced in older adults compared to younger. There was a general trend for reduced salivary responsiveness to stimuli in older adults compared to younger, but the heterogeneity in responses within the older age group may have obscured any significant differences between the groups. Indeed, large variability in responses in sensory testing of older adults has also been described in previous studies (Boesveldt et al. 2018).

Changes in extensional rheology of saliva, in response to oral stimuli, may also represent responsiveness to taste, odour and TRP agonists. Stimulating compounds may activate different salivary glands, secreting saliva with varying concentrations of mucin, which are responsible for the spinnbarkeit or stringiness of saliva (Inoue et al. 2008). In younger adults, caffeine and menthol stimulated saliva with reduced extensional rheology compared to water rinsing alone. Meanwhile, capsaicin stimulated a significant increase in salivary viscoelasticity, in agreement with previous studies (Houghton et al. 2017). However, the finding of reduced elasticity of menthol stimulated saliva is in contrast with other reports. This may be due to menthol having a transient effect, inducing increased viscoelasticity only in the first minute following stimulation, whereas in this study saliva was collected continuously for a 2 minute period (Houghton et al. 2017). In older adults there were no such changes in salivary viscoelasticity, in response to oral stimulants. This could be due to reduced responsiveness to stimulation or to age-related reduction in secretion from the mucin producing salivary glands – submandibular and sublingual (Affoo et al. 2015).

The results from this study may be limited by the low number of compounds tested and the fact that different concentrations were not assessed for each compound. However, the study design was kept simple to reduce participant fatigue and confusion, particularly in the older age group. Furthermore, concentrations of tastants found in foods are generally much higher than detection-threshold level, so determining supra-

threshold concentrations may be more relevant to the *in vivo* situation during food consumption (Stevens and Cain 1993, Methven et al. 2012). Never the less, perceptions of suprathreshold concentrations of tastants, presented as aqueous solutions, do not always correlate with those presented in foodstuffs (Mojet et al. 2003). Therefore, testing sensitivity to compounds dissolved in water, as opposed to testing food products, where synergy between flavours, texture and taste compounds may affect sensation, poses a limitation of this study.

A questionnaire was used to identify altered food behaviours, which might result from sensory loss in older adults. A higher, albeit statistically in-significant, proportion of older adults admitted to adding more salt to food over the past 5 years compared to younger adults. Tentatively, this may represent a negative effect of taste loss on nutrition and health, as salt consumption is linked to a range of diet-related diseases including hypertension (He and MacGregor 2009). The food behaviour questionnaire used in this study may not be fully representative of the population in general, because compliance with the questionnaire was low and because re-call relies on memory, which may lead to in-accurate results.

7.2 Age-Related Alterations in Salivary Properties

A reduction in salivary flow rate may be associated with ageing, although this is strongly influenced by increased use of medications with age (Affoo et al. 2015). However, the effect of ageing on salivary rheology and composition is less well studied. In chapter 3, a significant reduction in extensional rheology of UWMS from older adults, compared to younger adults, was shown. Previously, an increase in salivary relaxation times was shown in UWMS of older adults, suggesting an increase in viscoelasticity (Kazakov et al. 2009, Zussman et al. 2007). However, this measurement of salivary surface rheology is likely not comparable to the measurement of spinnbarkeit conducted in this study. Meanwhile, analysis of UWMS viscosity showed that there were no age-related changes. This was correlated to the observed changes in mucin composition of saliva in older adults. Levels of MUC7, responsible for salivary viscoelasticity, were significantly lower while levels of MUC5B, related to salivary viscosity, were not significantly altered in older subjects (Inoue et al. 2008, Ligtenberg et al. 2016). Further analysis of salivary mucin highlighted additional age-related alterations, with reduced sialylation of MUC7 in older adults. This suggests that MUC7 may be subject to degradation in the oral cavity of older adults. This may be a

consequence of impaired oral health and increased levels of mucin degrading bacteria, such as *Streptococci*, which have been shown to occur in older adults, in previous studies (Köhler and Persson 1991, Hunt et al. 1992, Griffin et al. 2012, Petersen and Yamamoto 2005, Katoh et al. 1997). Indeed Baughan et al (2002), demonstrated a correlation between levels of *Streptococcus mutans* and MUC7 glycosylation in older adults (Baughan et al. 2000). MUC7 de-sialylation and degradation in saliva of older adults may in turn lead to impaired mucosal defences and reduced lubrication of the oral cavity, which could lead to damage to the taste buds, resulting in a reduction in taste sensitivity. This may also affect dissolution of taste compounds and retention of taste and odorants in the oral cavity. Muco-adhesive properties of UWMS from older adults were also shown to be impaired, with reduced binding of MUC5B to oral epithelial cells *in vitro*.

The pH of all SWMS was significantly higher compared to UWMS in older adults, while in younger adults only menthol, capsaicin and MSG stimulation led to significantly higher pH. This supports the idea of greater proportions of parotid gland secretion in older adults, compared to younger adults, as parotid saliva has a greater proportion of bicarbonate and therefore, a higher pH than submandibular/sublingual saliva. The mechanical action of mouth rinsing may be responsible for the observed effect of stimulation on salivary pH, in older adults, as mechanical stimulation preferentially stimulates the parotid gland (Stokes and Davies 2007). The fact that water rinsing, with no stimulant, also gave rise to higher pH in saliva from older adults, further supports this theory.

While total protein was relatively un-affected by ageing, levels of salivary proteins, previously linked to taste function, were reduced in older adults compared to younger. Carbonic anhydrase VI (CAVI) may act as a growth factor for taste bud renewal and was significantly reduced in UWMS from older adults, in this study (Shatzman and Henkin 1981). Although, it is important to note that reports on the role of salivary CAVI in taste dysfunction are inconsistent and may require further investigation (Schoeps et al. 2016, Feeney and Hayes 2014, Rodrigues et al. 2017). Poor dietary zinc status, which occurs frequently in older adults, has shown to be linked with both CAVI levels in saliva and reduced salivary flow, therefore zinc deficiency may play a role in these changes observed in older adults (Komai et al. 2000, Rhodus and Brown 1990). Cystatins are cysteine protease inhibitors, thought to result in reduced caffeine taste

sensation, due to a barrier effect of thicker saliva resulting from reduced protease activity (Dsamou et al. 2012, Morzel et al. 2014). In contrast, proteins from the cystatin family have been shown to stabilise sweet taste receptors in the active form, which may heighten sweet taste sensation (Tancredi et al. 2004, Temussi 2011). However, while levels of cystatin S were reduced in older adults, compared to younger, this did not reach statistical significance due to large variation between individuals. Reduced cystatin S may correlate with greater proteolysis in saliva. This may be influential on salivary MUC7 levels since MUC7 proteolysis increases following de-glycosylation, which was found to be greater (chapter 4) in saliva from older adults.

7.3 Development of an *In vitro* Model of Taste Receptor Activation for the Study of Saliva and Age-Related Changes in Bitter Taste

To determine the contribution of saliva to reduced taste and smell in older adults, saliva samples collected from both older and younger adults were used in an *in vitro* model of taste receptor activation. Given the observed changes in both saliva and oral sensation in older adults, in this study, it was proposed that saliva from older adults may impair or reduce tastant-receptor interactions. Firstly, an *in vitro* model was developed using oral epithelial cells, namely TR146 MUC1, which have been used previously as a model for the oral epithelium due to binding capability for salivary MUC5B (Ployon et al. 2016), and SCC090, derived from tongue epithelium. Oral cell lines were chosen to facilitate interaction between salivary mucin and MUC1 expressed by the cells, as demonstrated in chapter 4, and to mimic the *in vivo* environment of taste receptors.

To investigate a potential role for saliva in age related reduction of PTC sensitivity, shown in chapter 3, TR146 MUC1 cells were transiently transfected with the PTC/PROP receptor, TAS2R38 and a chimeric gustducin protein, Gα16Gust44 (Ueda et al. 2003). Such heterologous expression systems have previously been used to successfully model TAS2R38 receptor activation but only in human embryonic kidney cells (Meyerhof et al. 2010), which, although highly transfectable, may not be representative of the oral environment. Expression of TAS2R38 and Gα16Gust44 could be shown in TR146 and TR146 MUC1 cells, at the level of protein and mRNA, confirming reproducible, over-expression. Intracellular calcium responses were significantly higher in response to PTC in the co-transfected cells compared to mock (empty vector) transfected cells, indicating functional expression of

TAS2R38:Gα16Gust44. Although some constitutive expression of TAS2R38 was shown in TR146 cells, this did not translate to any significant receptor function.

Calcium signalling in response to PTC, in TAS2R38:Gα16Gust44 transfected TR146 MUC1 cells, was significantly reduced in the presence of saliva from older adults but was un-affected by saliva from younger adults. Corroborating previous studies which have shown that reduced salivary cystatin S may be linked with heightened taste sensation for caffeine bitterness (Dsamou et al. 2012, Morzel et al. 2014), there was a negative correlation between cystatin S in UWMS and the calcium response to PTC in the UWMS assay. Although the role for cystatin S has been previously linked with reduced proteolysis in saliva and thus more viscous saliva forming a barrier to taste compounds, there was no correlation between salivary rheology and the calcium responses to PTC in the cell model, in this study. Therefore, the role for cystatin S in taste receptor activation may occur independently of any effect on salivary rheology.

Despite total protein concentrations being similar in UWMS from both age groups, levels of individual proteins, analysed in this study, were reduced in older adults. It is therefore possible that other proteins, not assessed in this study, are also reduced in UWMS of older adults. This may include basic proline rich proteins (bPRPs), some of which can enhance TAS2R38 activation by altering the orientation of the agonist to enhance binding to the receptor (Melis et al. 2013a, Cabras et al. 2012).

In SCC090 cells, endogenous bitter taste receptor expression was first demonstrated. It was shown, in chapter 5, that the cells expressed the bitter receptors TAS2R10, TAS2R43 and TAS2R38. Functional experiments were conducted, and there were significant responses to both PTC, indicating functional TAS2R38 expression, and caffeine, possibly demonstrating TAS2R10/TAS2R43 activation. Incubating SCC090 cells with UWMS from younger adults led to significantly greater calcium responses to caffeine, compared with UWMS from older adults. Interestingly, UWMS from younger adults also led to increased caffeine responses than with buffer alone, implying a role for saliva in enhancing caffeine-receptor activation. Contrasting with the results from the PTC experiments, salivary cystatin S levels could be correlated positively with the caffeine response in SCC090 cells, in the presence of UWMS. It was speculatively proposed therefore, that cystatin S may stabilise the caffeine receptor in its active form, as previously shown for proteins with structural homology to cystatins, with sweet taste receptors (Tancredi et al. 2004, Temussi 2011). UWMS from younger adults, had

significantly higher levels of cystatin S compared to older adults, which may be linked with increased calcium responses to caffeine.

The structural variations of different bitter taste compounds may affect dissolution and transport to taste receptors in the presence of saliva. For example, caffeine is less lipophilic than PTC and older adults retain taste sensitivity to lipophilic compounds less well than to more polar compounds (Schiffman et al. 1994a), possibly because of interactions between salivary mucin and lipophilic molecules. Indeed, lipophilicity is linked with greater mucin binding of a compound (Larhed et al., 1997; Matthes et al., 1992; Norris and Sinko, 1997). Additionally, PTC contains a thiol group, which may participate in disulphide-thiol exchange reactions with the cysteine regions of mucin in saliva, forming disulphide bonds (Bernkop-Schnürch et al. 1999). The more likely formation of covalent bonds between thiol- compounds and mucin could enhance diffusion and increase availability of the compound for receptors at the mucosal surface (Bernkop-Schnürch et al. 2004, Dünnhaupt et al. 2011, Clausen and Bernkop- Schnürch 2000). As such, in UWMS from younger adults, MUC7 was positively correlated to PTC calcium responses in the TR146 MUC1 model. Degradation of MUC7 in saliva of older adults may therefore be responsible for a lack of correlation between MUC7 and *in vitro* PTC responses, but also for the reduced responses in the TAS2R38 cell model in the presence of older UWMS.

Meanwhile, caffeine is amphiphilic in nature and can act as a hydrotrope to enhance water solubility of other hydrophobic molecules, while concurrently increasing the solubility of caffeine itself (Cui 2010, Lim and Go 2000, Rasool et al. 1991). As such, caffeine may interact with hydrophobic regions of mucin to increase solubility and reduce diffusion time to the receptor. In older adults, where salivary mucin levels are reduced, such effects may be reduced and therefore responses to caffeine were greater with UWMS from younger adults.

7.4 Future Work

In the short term, expression of a wider range of taste receptors could be characterised in the SCC090 cell line, to assess the capacity of this cell line as a model for taste receptor activation. For completeness, suitable positive and negative controls should be used when characterising expression levels, for example use of over-expression cell lines as positive controls and a cell line which does not constitutively express taste receptors as a negative control. Indeed, use of inhibitors and/or siRNA knockout of taste receptor genes in SCC090 cell functional assays, would allow for identification of the source of calcium responses to taste compounds in these cells.

Further work could be directed towards development of an SCC090 cell line, stably expressing MUC1, which could increase binding of salivary mucin and thus improve the potential of this cell line, as a model of the tongue epithelium. In addition, the model developed in this thesis could be used to assess the effects of alterations in saliva on the activation of different taste receptors, using transient transfection of bitter, sweet and umami receptors in TR146 MUC1 cells. Furthermore, saliva samples from individuals with taste disorders of different aetiologies could also be used in conjunction with the model described here. This would allow for further understanding of the role of saliva in taste dysfunction generally.

Additionally, saliva samples from older adults could be assessed for a wider range of proteins, to provide greater insight into which may be involved in taste receptor activation. This could involve more extensive immuno-blot analyses, as conducted in this thesis, or proteomics techniques such as HPLC-MS. For instance, since older adults had reduced sensitivity to PTC and their saliva also reduced calcium responses to PTC *in vitro*, it would be interesting to assess levels of the bPRPs, II-2 and Ps-1. Both proteins have been shown previously to be higher in individuals with TAS2R38 taster status compared to non-tasters and could therefore be implicated in age-related decline of PTC sensitivity.

Changes in mucin glycosylation were shown in this study but the source of these changes was not identified. Therefore, an interesting line of study would be to investigate the oral microbiome in older adults, specifically in relation to mucin degrading bacteria. Furthermore, strategies to improve mucin status in saliva of older adults could enhance the quality of saliva in an age group at risk of xerostomia and

related oral disorders. If changes in oral bacteria were identified as a causal factor for mucin degradation, possible interventions could include improving oral health status or pre/probiotic treatments to enhance growth of beneficial flora and reduce growth of pathogenic species. Also, development of saliva substitutes containing mucin or a mucin like substance may enhance the quality of saliva in older adults and could improve oral health and taste function. Indeed, the results from this study demonstrate the need for saliva substitutes which mimic the physical properties of saliva and thus the focus for development of such products should be on the quality of saliva rather than just increasing the quantity.

The use of differential dynamic microscopy (DDM) is presented in this thesis as a novel technique for measurement of viscosity in low volume saliva samples. In order to confirm the results, a comparative study should be undertaken to compare traditional methods of salivary viscosity measurement, such as cone and plate rheology, with DDM. Also, further work is needed to assess the contribution of viscoelasticity on DDM measurements, to ensure that viscosity values are not affected by surface rheology of saliva.

To identify the mechanism behind altered receptor activation in the presence of saliva, several strategies could be employed. Firstly, to investigate whether TAS2R receptors are down-regulated or internalised upon agonist stimulation, as proposed in chapter 6, a time-course study could be used. This would involve characterising gene expression, using RT-PCR or microarray, following agonist stimulation at different time points. Furthermore, confocal microscopy could be used to visualise cellular location of expressed TAS2R in individual cells, using florescent labelling of the receptor of interest. One method of tagging the receptor fluorescently could be to use CRISPR/Cas-mediated genome engineering, with RNA templates for the relevant taste receptor gene(s) (Leonetti et al. 2016). Alternatively, when transfecting TAS2R proteins into oral epithelial cells, a GFP tag could be ligated into the plasmid. This would allow visualisation of receptor localisation within the cell, after addition of the agonist.

To allow for identification of specific salivary proteins involved in altered *in vitro* taste receptor activation, one could utilise saliva from individual glandular sources. For example, using parotid saliva alone on the cell model and comparing with whole mouth or submandibular/sublingual saliva, would provide insight into the role for mucin in taste receptor activation. Furthermore, it may be possible to “supplement” saliva from

older adults with proteins of interest to observe which, if any, proteins increase tastant-receptor activation. Purified mucins are commercially available and could be used for this purpose. In addition, previous studies have also purified Ps-1 and II-2 proteins from WMS and used them to supplement WMS *in vivo*, in order to demonstrate the effect on PROP taste sensitivity (Melis et al. 2013a). This can be achieved by first concentrating the acidic soluble portion, using lyophilization, and then utilising gel filtration to separate protein fractions, followed by HPLC-MS to identify the fractions (Melis et al. 2013a). Cystatin S is another protein proposed to have a role in taste receptor activation, in this study, and could also be purified using gel-filtration chromatography (Isemura et al. 1986). It would also be of interest to see whether supplementing saliva with cystatin S, resulted in any changes in the rheological properties of saliva. This would provide a greater understanding of the mechanism behind the association between taste function and salivary cystatin S.

Moreover, *in vivo* studies using animal models could further demonstrate the relevance of saliva in taste function. Saliva from older adults could be applied in animal models to investigate the effects on behavioural and neuronal responses to a range of taste compounds. Alternatively, tongue tissue from rats or mice could be used in place of the cell model, for calcium response assays, to assess the effect of saliva on taste responses. Indeed, as described above, supplementing saliva with various individual proteins would provide an understanding of the mechanisms behind altered taste receptor activation in the presence of WMS. This could also be applied to animal studies, to demonstrate any effects of salivary supplementation on taste function, *in vivo*.

In the longer term, longitudinal studies of taste function and related food behaviours would provide greater understanding of the nature of age-related sensory loss and the effect it has on nutrition and health.

7.5 Final Remarks

Nutrition and health of the ageing population is known to deteriorate, in part due to loss of taste and smell function which negatively affect appetite. In the past, taste testing has focussed largely on subjective measures such as perceptions, but these are affected by individual experience and in the case of older adults, particularly, by cognitive function. Therefore, measurement of salivary response to taste stimulation was shown in this study and may be used in future sensory research as an additional method of assessing sensory responses.

The main findings from this thesis are:

1) Salivary rheology is altered in older adults and contributes to taste loss. Reduced levels and sialylation of MUC7 may lead to reduced visco-elasticity and impaired muco-adhesion of saliva from older adults. Loss of mucin function in saliva, due to de-glycosylation occurring in ageing, may also affect oral health by reducing mucosal defences in the oral cavity and in turn, impair sensitivity to oral stimulants. In addition, changes in the levels of specific salivary proteins such as cystatins, could also play a role in reduced tastant-receptor interactions in older adults.
2) Salivary reflex response to oral stimulation provides an objective measure of function in older adults. This is a useful method for assessing oral sensitivity in older adults, where cognitive impairment and fatigue may bias self-reported perceptions.
3) Transfection of taste receptors into oral epithelial cells provides a model for taste receptor activation. In addition, the results presented in this thesis demonstrate that saliva can affect taste receptor activation *in vitro*, therefore future studies utilising cell-based models for taste, should consider the role of saliva.
4) Saliva from older adults facilitates bitter taste receptor activation less well than saliva from younger adults. The causes of taste dysfunction in older adults are largely un-known, but this thesis presents support for a role of saliva in facilitating activation of taste receptors, which forms the initial stage of taste signalling.

Therefore, this research provides a platform of information regarding the properties of human saliva in ageing, which may aid in future research, towards development of a

more bio-relevant saliva substitute, which may improve taste function and quality of life for older adults. In addition, a robust, analytical approach to measuring tastant-receptor interaction was developed which may be used in future applications, as a biologically relevant system for taste evaluation *in vitro*.

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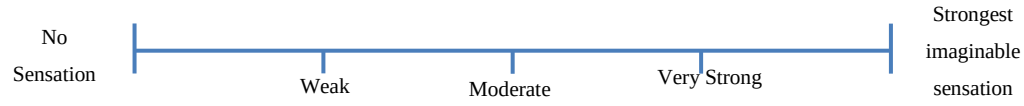
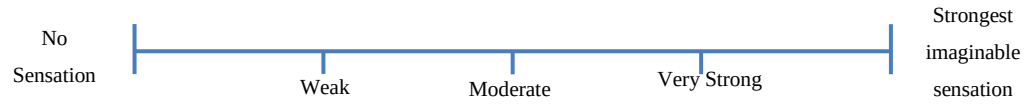
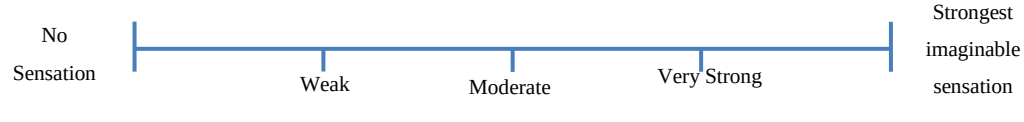
9 Appendices

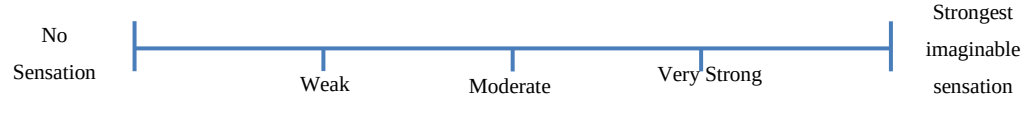
9.1 Appendix 1. Labelled scale used to assess taste perceptions

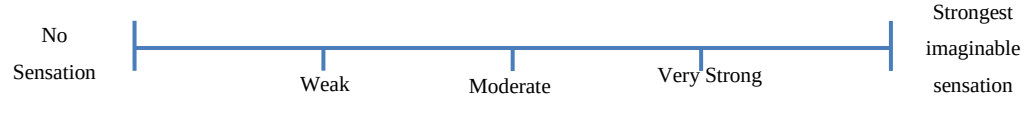
How intense did you find the taste/smell of the solution?

1. 

2. 

3. 

4. 

5. 

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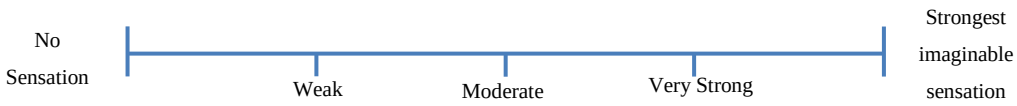
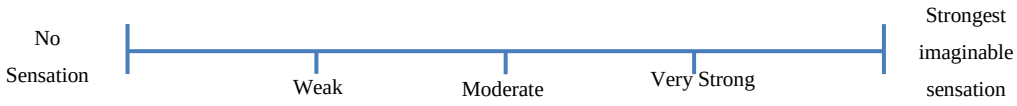


PTC Taster Strip



9.2 Appendix 2. Questionnaire about food behaviour and beliefs on taste and smell loss

Instructions to Participants

1. Thank you for agreeing to complete a further questionnaire in this study. In this part we would like to collect some demographic data about participants, you do not have to answer these questions and your answers will remain confidential and anonymous.

How many medications do you take currently per day (prescribed medications issued by your GP)?

How many over the counter or alternative medications (including vitamins, supplements or complimentary medicines) do you take per day?

Please write the number of medications you take daily from the following categories:

Anti-depressants

Anti-hypertensives (blood pressure medications)

Anti-hyperlipidemic (cholesterol medications)

Anti-Anxiety

Narcotics/Painkillers

Diuretics (water tablets)

Antacids (acid reflux or heart burn medications)

What is the highest level of qualification you hold? Please tick the box that applies.

No qualifications ☐

O Grade, Standard Grade, Access 3 Cluster, Intermediate 1 or 2, GCSE, CSE, Senior Certificate or equivalent; GSVQ Foundation or Intermediate, SVQ level 1 or 2, SCOTVEC Module, City and Guilds Craft or equivalent; Other school qualifications not already mentioned (including foreign qualifications) ☐

SCE Higher Grade, Higher, Advanced Higher, CSYS, A Level, AS Level, Advanced Senior Certificate or equivalent; GSVQ Advanced, SVQ level 3, ONC, OND, SCOTVEC National Diploma, City and Guilds Advanced Craft or equivalent ☐

HNC, HND, SVQ level 4 or equivalent; Other post-school but pre-Higher Education qualifications not already mentioned (including foreign qualifications) ☐

Degree, Postgraduate qualifications, Masters, PhD, SVQ level 5 or equivalent; Professional qualifications (for example, teaching, nursing, accountancy); Other Higher Education qualifications not already mentioned (including foreign qualifications) ☐

Which race/ethnicity best describes you? (Please tick any applicable or write other)

A : White

English/Welsh/Scottish/Northern Irish/British ☐

Irish ☐

Gypsy or Irish Traveller ☐

Any other White background (please write in) ☐

B : Mixed/Multiple Ethnic Groups

White and Black Caribbean ☐

White and Black African ☐

White and Asian ☐

Any other mixed/multiple ethnic background (please write in) ☐

C : Asian or Asian British

Indian ☐

Pakistani ☐

Bangladeshi ☐

Chinese ☐

Any other Asian background (please write in) ☐

D : Black/African/Caribbean/Black British

Caribbean ☐

African ☐

Any other Black/African/Caribbean background (please write in) ☐

E : Other ethnic group

Arab ☐

Any other ethnic group (please write in) ☐

Not stated ☐

2. In this part, we are interested in finding out about how your lifestyle might have changed over the recent past. There are no right, or wrong answers and your answers will remain confidential and anonymous.

Remembering information about our past can be tricky. That is because things we remember about the past tend to blend in and get mixed with everyday life. To try and tease apart the 'now' from 'the past' we would like you to take a few moments to try and remember where you were the summer of the London Olympics. Take a few moments to refresh your memory- try and think where you spent that summer, what the weather was like, what daily activities you did, how much of the Olympics you followed.

Once you are confident you can think back to that summer, please tell us how confident you are about remembering that summer. Please use the following scale and tick the box under the number that applies to you. Remember there are no right or wrong answers.

1	***2***	***3***	***4***	***5***
Not at all confident- that summer is very blurry in my mind				***Extremely confident – I remember that summer really well***

The next section of the questionnaire asks you about some everyday life behaviours that you might be undertaking NOW.

We would like you to think about the past 7 days and compare that week with the summer of the London Olympics*. Then, we would like you to tell us, how what you do now, is the same or different from what you did during the London Olympics. Please do this by ticking the box that applies.*

For example:-

Thinking about the past 7 days vs. the summer of the London Olympics….

I have lost weight

Yes	*No*	*I cannot remember*
	v	

Please complete the boxes for the items that follow. Remember there are no right or wrong answers.

I have taken up a new hobby

Yes	*No*	*I cannot remember*

I have cut down on my alcohol intake.

Yes	*No*	*I cannot remember*

I have been adding more salt to my food.

Yes	*No*	*I cannot remember*

I have been eating fewer vegetables.

Yes	*No*	*I cannot remember*

I have been eating more sugary foods such as cakes, biscuits, chocolate and sweets.

Yes	*No*	*I cannot remember*

I have found it easier to sit back and relax.

Yes	*No*	*I cannot remember*

I have been going on more mini breaks / holidays.

Yes	*No*	*I cannot remember*

I have lost interest in my appearance

Yes	*No*	*I cannot remember*

I have been doing more physical exercise

Yes	*No*	*I cannot remember*

The smell of food/drinks is not as strong e.g. The smell of freshly baked bread

Yes	*No*	*I cannot remember*

I have visited my GP or hospital more often

Yes	*No*	*I cannot remember*

I have been adding new foods to my diet

Yes	*No*	*I cannot remember*

I have felt more sad, down or disinterested in life

Yes	*No*	*I cannot remember*

I have been having more spicy food

Yes	*No*	*I cannot remember*

I have found it easier to concentrate

Yes	*No*	*I cannot remember*

I have lost pleasure in eating

Yes	*No*	*I cannot remember*

The taste of food/drinks is not as strong e.g. The taste of salt in soup or the taste of sugar in tea

Yes	*No*	*I cannot remember*

9.3 Appendix 3. Plasmid Maps

Plasmid name: **hTAS2R38 PAV**

Date: 10.10.2011

Vector: pcDNA5FRT PM

Insert: hTAS2R38 PAV

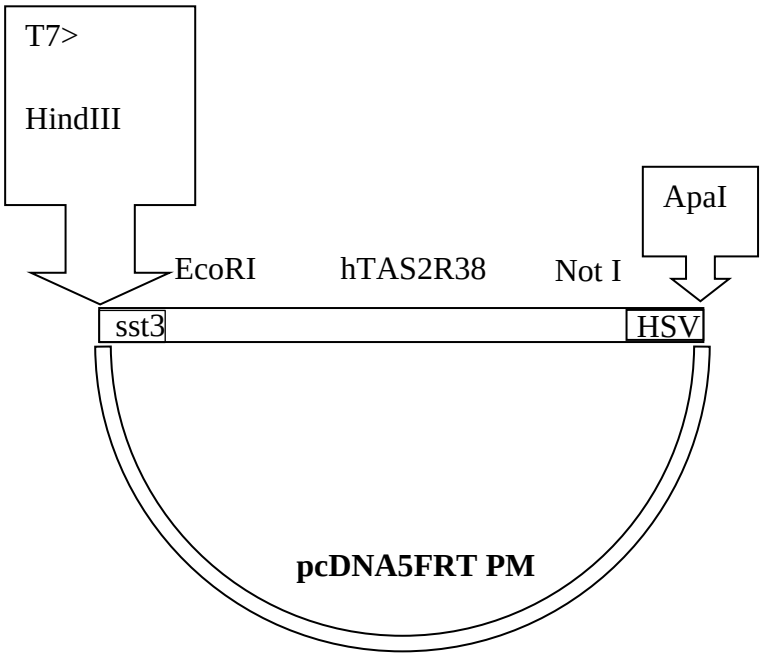
Origin: human

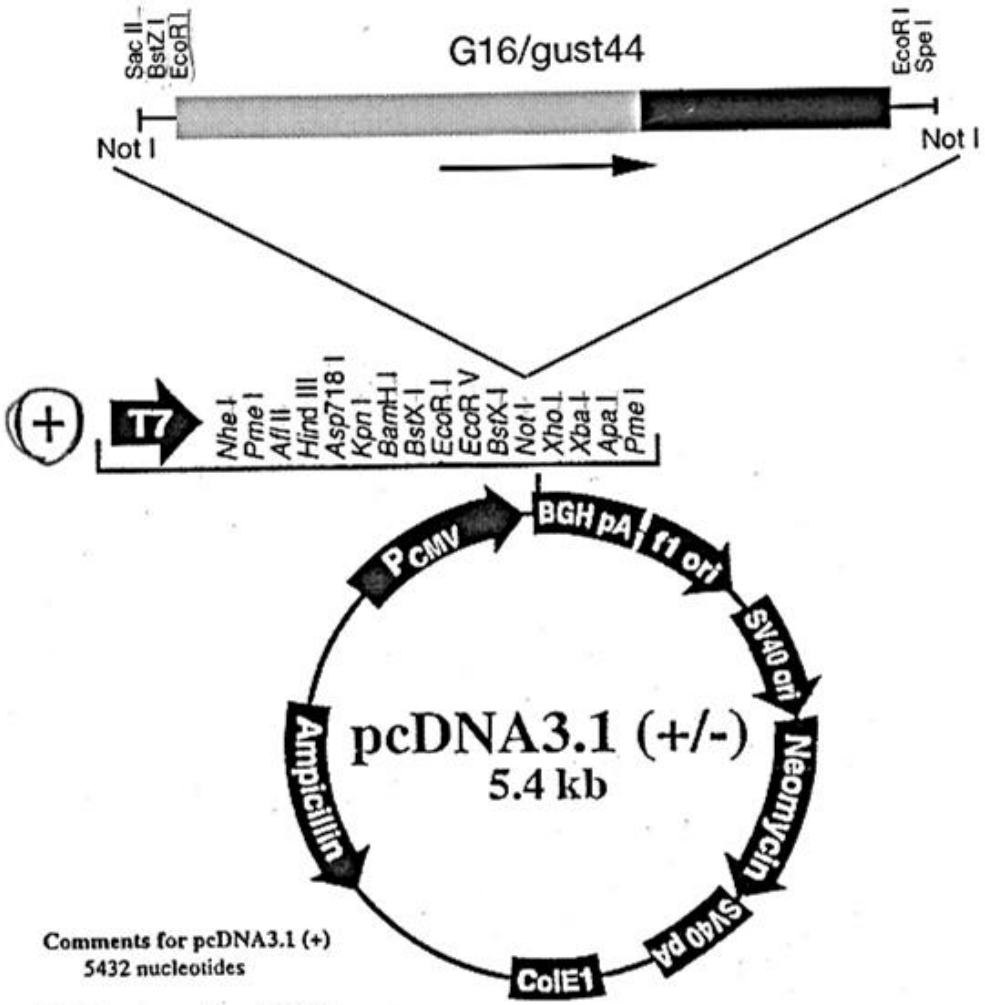
Bacteria: XL1-blue

Resistance: Hygromycin (eukaryote), Ampicillin (prokaryote)

Sequence: T7-MCS-pcDNA3.1/BGH

Comments: CMV Promotor, BGH poly(A), SV40 poly(A), pUC Ori, FRT site





Comments for pcDNA3.1 (+)
5432 nucleotides

CMV promoter: bases 209-863
T7 promoter/priming site: bases 863-882
Multiple cloning site: bases 895-1010
pcDNA3.1/BGH reverse priming site: bases 1022-1039
BGH polyadenylation signal: bases 1021-1235
f1 origin of replication: bases 1298-1711
SV40 promoter and origin: bases 1776-2101
Neomycin resistance gene: bases 2137-2931
SV40 polyadenylation signal: bases 2947-3186
ColE1 origin: bases 3618-4291
Ampicillin resistance gene: bases 4436-5297

9.4 Appendix 4. Raw data not normalised to water rinse

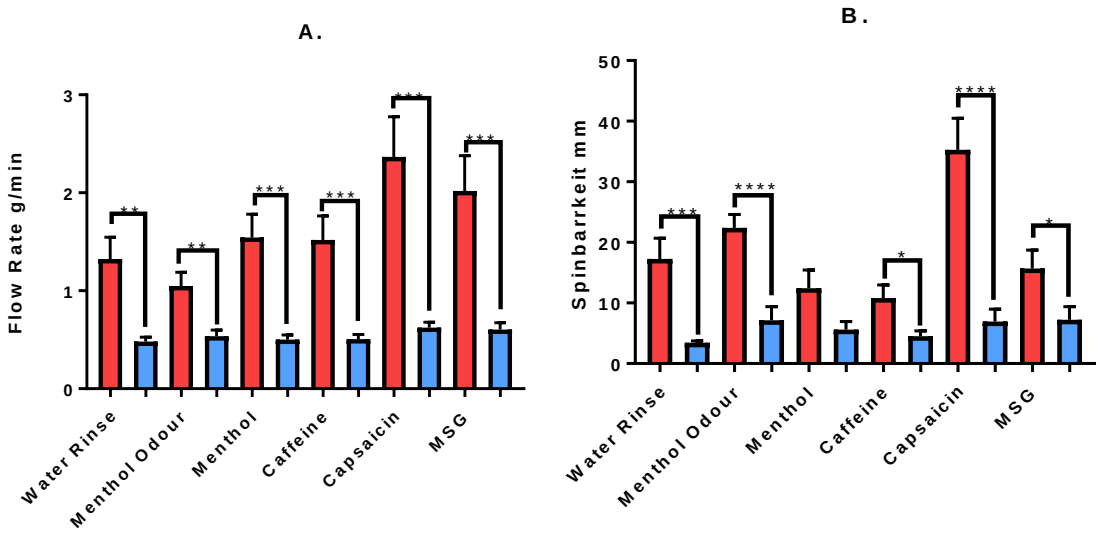


Figure 9-1 SWMS flow rate and spinnbarkeit in older and younger adults not normalised to water rinse. A. Mean (+/-SEM) WMS flow rate following 1 minute of 1ml mouth rinse of taste and TRP agonists or 1-minute smelling of menthol in younger (18-30 years) and older (60+ years) subjects. 18-30 years old shown in red (N=31), 60+ years old shown in blue (N=25). B. Mean (+/-SEM) Spinnbarkeit (ER) of WMS following 1 minute of 1ml mouth rinse of taste or TRP agonist or 1-minute smelling of menthol, assessed using the NevaMeter. 18-30 years old shown in red (N=31), 60+ years old shown in blue (N=25). Tested for statistical significance using independent students T-Test (P = < 0.05 * P = < 0.01 ** P = < 0.001 *** P=<0.001 ****).

Since there was a significant difference in flow rate and Spinnbarkeit of water rinse evoked SWMS between older and younger adults, data was subsequently normalised to water rinse SWMS to allow for effects occurring solely from stimulant compounds to be seen.

9.5 Appendix 5. Correlations between UWMS pH and salivary parameters

Table 9-1 Kendall's tau b correlation between UWMS pH and UWMS/SWMS flow rates

Correlations

	Age of Participant			pH resting saliva
Kendall's tau_b	18-30 Years Old	pH resting saliva	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	14
		Resting flow rate	Correlation Coefficient	.011
			Sig. (2-tailed)	.956
			N	14
		Water rinse flow rate	Correlation Coefficient	.167
			Sig. (2-tailed)	.410
			N	14
		Menthol odour flow rate	Correlation Coefficient	.056
			Sig. (2-tailed)	.784
			N	14
		Menthol flow rate	Correlation Coefficient	.033
			Sig. (2-tailed)	.869
			N	14
		Caffeine flow rate	Correlation Coefficient	.100
			Sig. (2-tailed)	.621
			N	14
		MSG flow rate	Correlation Coefficient	.233
			Sig. (2-tailed)	.249
			N	14
		Capsaicin flow rate	Correlation Coefficient	.144
			Sig. (2-tailed)	.475
			N	14
	60+ Years Old	pH resting saliva	Correlation Coefficient	1.000
			Sig. (2-tailed)	.

	N	10
Resting flow rate	Correlation Coefficient	-.200
	Sig. (2-tailed)	.421
	N	10
Water rinse flow rate	Correlation Coefficient	-.156
	Sig. (2-tailed)	.531
	N	10
Menthol odour flow rate	Correlation Coefficient	-.422
	Sig. (2-tailed)	.089
	N	10
Menthol flow rate	Correlation Coefficient	-.244
	Sig. (2-tailed)	.325
	N	10
Caffeine flow rate	Correlation Coefficient	-.333
	Sig. (2-tailed)	.180
	N	10
MSG flow rate	Correlation Coefficient	-.333
	Sig. (2-tailed)	.180
	N	10
Capsaicin flow rate	Correlation Coefficient	.022
	Sig. (2-tailed)	.929
	N	10

**. Correlation is significant at the 0.01 level (2-tailed).

Table 9-2 Kendall's tau b correlation between UWMS pH and spinnbarkeit of UWMS and SWMS

Correlations

	Age of Participant			pH resting saliva
Kendall's tau_b	18-30 Years Old	pH resting saliva	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	14
		Spinnbarkeit resting	Correlation Coefficient	-.011
			Sig. (2-tailed)	.956
			N	14
		Spinnbarkeit water rinse	Correlation Coefficient	-.122
			Sig. (2-tailed)	.546
			N	14
		Spinnbarkeit menthol odour	Correlation Coefficient	-.278
			Sig. (2-tailed)	.170
			N	14
		Spinnbarkeit menthol	Correlation Coefficient	.100
			Sig. (2-tailed)	.621
			N	14
		Spinnbarkeit caffeine	Correlation Coefficient	-.358
			Sig. (2-tailed)	.078
			N	14
		Spinnbarkeit MSG	Correlation Coefficient	-.100
			Sig. (2-tailed)	.621
			N	14
		Spinnbarkeit Capsaicin	Correlation Coefficient	.167
			Sig. (2-tailed)	.410
			N	14
	60+ Years Old	pH resting saliva	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	10
		Spinnbarkeit resting	Correlation Coefficient	.022
			Sig. (2-tailed)	.929
			N	10
		Spinnbarkeit water rinse	Correlation Coefficient	.244
			Sig. (2-tailed)	.325
			N	10
		Spinnbarkeit menthol odour	Correlation Coefficient	-.156
			Sig. (2-tailed)	.531
			N	10
		Spinnbarkeit menthol	Correlation Coefficient	.135

	Sig. (2-tailed)	.590
	N	10
Spinnbarkeit caffeine	Correlation Coefficient	-.022
	Sig. (2-tailed)	.929
	N	10
Spinnbarkeit MSG	Correlation Coefficient	.022
	Sig. (2-tailed)	.929
	N	10
Spinnbarkeit Capsaicin	Correlation Coefficient	.135
	Sig. (2-tailed)	.590
	N	10

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 9-3 Kendall's tau b correlation between pH and UWMS composition

Correlations

	Age of Participant			pH resting saliva
Kendall's tau_b	18-30 Years Old	pH resting saliva	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	14
		Total Protein Resting Saliva	Correlation Coefficient	-.256
			Sig. (2-tailed)	.207
			N	14
		MUC5B Resting	Correlation Coefficient	.122
			Sig. (2-tailed)	.546
			N	14
		Muc7 Resting	Correlation Coefficient	-.056
			Sig. (2-tailed)	.784
			N	14
		Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	-.189
			Sig. (2-tailed)	.351
			N	14
		Cystatin S in resting saliva	Correlation Coefficient	-.278
			Sig. (2-tailed)	.170
			N	14
	60+ Years Old	pH resting saliva	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	10
		Total Protein Resting Saliva	Correlation Coefficient	.067
			Sig. (2-tailed)	.788
			N	10
		MUC5B Resting	Correlation Coefficient	-.022
			Sig. (2-tailed)	.929
			N	10
		Muc7 Resting	Correlation Coefficient	.022
			Sig. (2-tailed)	.929
			N	10
		Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	.022
			Sig. (2-tailed)	.929
			N	10
		Cystatin S in resting saliva	Correlation Coefficient	-.067
			Sig. (2-tailed)	.788
			N	10

**. Correlation is significant at the 0.01 level (2-tailed).

9.6 Appendix 6. Correlations between medication use and taste function/salivary parameters

Table 9-4 Kendall's tau b correlation between medication use and salivary spinnbarkeit

Correlations

				Prescription Med	OTC Med
	Age of Participant				
Kendall's tau_b	18-30 Years Old	PrecriptionMed	Correlation Coefficient	1.000	-.189
			Sig. (2-tailed)	.	.592
			N	8	8
		OTCMed	Correlation Coefficient	-.189	1.000
			Sig. (2-tailed)	.592	.
			N	8	8
		Spinnbarkeit resting	Correlation Coefficient	-.218	.041
			Sig. (2-tailed)	.505	.893
			N	8	8
		Spinnbarkeit water rinse	Correlation Coefficient	-.218	.206
			Sig. (2-tailed)	.505	.503
			N	8	8
		Spinnbarkeit menthol odour	Correlation Coefficient	.000	-.041
			Sig. (2-tailed)	1.000	.893
			N	8	8
		Spinnbarkeit menthol	Correlation Coefficient	-.655*	.206
			Sig. (2-tailed)	.046	.503
			N	8	8
		Spinnbarkeit caffeine	Correlation Coefficient	-.218	.206
			Sig. (2-tailed)	.505	.503
			N	8	8
		Spinnbarkeit MSG	Correlation Coefficient	-.327	.454
			Sig. (2-tailed)	.317	.140
			N	8	8
		Spinnbarkeit Capsaicin	Correlation Coefficient	-.546	.041
			Sig. (2-tailed)	.096	.893
			N	8	8
	60+ Years Old	PrecriptionMed	Correlation Coefficient	1.000	.146
			Sig. (2-tailed)	.	.602
			N	11	11
		OTCMed	Correlation Coefficient	.146	1.000

	Sig. (2-tailed)	.602	.
	N	11	11
Spinnbarkeit resting	Correlation Coefficient	.261	.000
	Sig. (2-tailed)	.291	1.000
	N	11	11
Spinnbarkeit water	Correlation Coefficient	-.020	-.476
rinse	Sig. (2-tailed)	.935	.070
	N	11	11
Spinnbarkeit menthol	Correlation Coefficient	.342	.423
odour	Sig. (2-tailed)	.167	.107
	N	11	11
Spinnbarkeit menthol	Correlation Coefficient	.325	-.027
	Sig. (2-tailed)	.193	.920
	N	11	11
Spinnbarkeit caffeine	Correlation Coefficient	.261	.053
	Sig. (2-tailed)	.291	.840
	N	11	11
Spinnbarkeit MSG	Correlation Coefficient	.744**	.000
	Sig. (2-tailed)	.003	1.000
	N	11	11
Spinnbarkeit Capsaicin	Correlation Coefficient	.325	.080
	Sig. (2-tailed)	.193	.762
	N	11	11

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 9-5 Kendall's tau b correlation between medication use and salivary flow rate

Correlations

	Age of Participant			PrecriptionMed	OTCMed
Kendall's tau_b	18-30 Years Old	PrecriptionMed	Correlation Coefficient	1.000	-.189
			Sig. (2-tailed)	.	.592
			N	8	8
		OTCMed	Correlation Coefficient	-.189	1.000
			Sig. (2-tailed)	.592	.
			N	8	8
		Resting flow rate	Correlation Coefficient	-.546	-.041
			Sig. (2-tailed)	.096	.893
			N	8	8
		Water rinse flow rate	Correlation Coefficient	-.327	-.289
			Sig. (2-tailed)	.317	.348
			N	8	8
		Menthol odour flow rate	Correlation Coefficient	-.327	-.454
			Sig. (2-tailed)	.317	.140
			N	8	8
		Menthol flow rate	Correlation Coefficient	-.218	-.454
			Sig. (2-tailed)	.505	.140
			N	8	8
		Caffeine flow rate	Correlation Coefficient	-.218	-.454
			Sig. (2-tailed)	.505	.140
			N	8	8
		MSG flow rate	Correlation Coefficient	-.218	-.454
			Sig. (2-tailed)	.505	.140
			N	8	8
		Capsaicin flow rate	Correlation Coefficient	.109	-.536
			Sig. (2-tailed)	.739	.081
			N	8	8
	60+ Years Old	PrecriptionMed	Correlation Coefficient	1.000	.146
			Sig. (2-tailed)	.	.602
			N	11	11
		OTCMed	Correlation Coefficient	.146	1.000
			Sig. (2-tailed)	.602	.
			N	11	11
		Resting flow rate	Correlation Coefficient	-.304	-.107
			Sig. (2-tailed)	.222	.686
			N	11	11
		Water rinse flow rate	Correlation Coefficient	.020	.159
			Sig. (2-tailed)	.935	.546

	N	11	11
Menthol odour flow rate	Correlation Coefficient	.203	.454
	Sig. (2-tailed)	.416	.086
	N	11	11
Menthol flow rate	Correlation Coefficient	-.261	-.264
	Sig. (2-tailed)	.291	.314
	N	11	11
Caffeine flow rate	Correlation Coefficient	-.181	.212
	Sig. (2-tailed)	.465	.421
	N	11	11
MSG flow rate	Correlation Coefficient	.020	.317
	Sig. (2-tailed)	.935	.227
	N	11	11
Capsaicin flow rate	Correlation Coefficient	-.221	.159
	Sig. (2-tailed)	.372	.546
	N	11	11

**. Correlation is significant at the 0.01 level (2-tailed).

Table 9-6 Kendall's tau b correlation between medication use and taste perception

Correlations

	Age of Participant			PrecriptionMed	OTCMed
Kendall's tau_b	18-30 Years Old	PrecriptionMed	Correlation Coefficient	1.000	-.189
			Sig. (2-tailed)	.	.592
			N	8	8
		OTCMed	Correlation Coefficient	-.189	1.000
			Sig. (2-tailed)	.592	.
			N	8	8
		Taste perception water rinse 1-10	Correlation Coefficient	.231	-.175
			Sig. (2-tailed)	.497	.584
			N	8	8
		Taste perception menthol odour 1-10	Correlation Coefficient	.510	.086
			Sig. (2-tailed)	.129	.786
			N	8	8
		Taste perception menthol 1-10	Correlation Coefficient	.000	.535
			Sig. (2-tailed)	1.000	.096
			N	8	8
		Taste perception caffeine 1-10	Correlation Coefficient	.453	-.214
			Sig. (2-tailed)	.177	.497
			N	8	8
		Taste perception MSG 1-10	Correlation Coefficient	-.667*	.504
			Sig. (2-tailed)	.044	.105
			N	8	8
		Taste perception capsaicin 1-10	Correlation Coefficient	.000	.186
			Sig. (2-tailed)	1.000	.571
			N	8	8
	60+ Years Old	PrecriptionMed	Correlation Coefficient	1.000	.146
			Sig. (2-tailed)	.	.602
			N	11	11
		OTCMed	Correlation Coefficient	.146	1.000
			Sig. (2-tailed)	.602	.

	N	11	11
Taste perception water rinse 1-10	Correlation Coefficient	-.457	.057
	Sig. (2-tailed)	.080	.837
	N	11	11
Taste perception menthol odour 1-10	Correlation Coefficient	-.495	-.057
	Sig. (2-tailed)	.055	.836
	N	11	11
Taste perception menthol 1-10	Correlation Coefficient	.170	.336
	Sig. (2-tailed)	.508	.219
	N	11	11
Taste perception caffeine 1-10	Correlation Coefficient	-.348	.296
	Sig. (2-tailed)	.165	.266
	N	11	11
Taste perception MSG 1-10	Correlation Coefficient	-.609*	-.229
	Sig. (2-tailed)	.019	.407
	N	11	11
Taste perception capsaicin 1-10	Correlation Coefficient	-.274	.139
	Sig. (2-tailed)	.284	.610
	N	11	11

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Correlations

	Age of Participant			PrecriptionMed	OTCMed
Kendall's tau_b	18-30 Years Old	PrecriptionMed	Correlation Coefficient	1.000	-.189
			Sig. (2-tailed)	.	.592
			N	8	8
		OTCMed	Correlation Coefficient	-.189	1.000
			Sig. (2-tailed)	.592	.
			N	8	8
		is taste sensitivity low or high	Correlation Coefficient	.149	.676
			Sig. (2-tailed)	.693	.055

		N	8	8
60+ Years Old	PrecriptionMed	Correlation Coefficient	1.000	.146
		Sig. (2-tailed)	.	.602
		N	11	11
	OTCMed	Correlation Coefficient	.146	1.000
		Sig. (2-tailed)	.602	.
		N	11	11
	is taste sensitivity low or high	Correlation Coefficient	.030	.040
		Sig. (2-tailed)	.916	.896
		N	11	11

Table 9-7 Kendall's tau b correlation between medication use and salivary chemical and compositional properties

Correlations

	Age of Participant			PrecriptionMed	OTCMed
Kendall's tau_b	18-30 Years Old	PrecriptionMed	Correlation Coefficient	1.000	-.189
			Sig. (2-tailed)	.	.592
			N	8	8
		OTCMed	Correlation Coefficient	-.189	1.000
			Sig. (2-tailed)	.592	.
			N	8	8
		Total Protein Resting Saliva	Correlation Coefficient	.000	-.124
			Sig. (2-tailed)	1.000	.688
			N	8	8
		MUC5B Resting	Correlation Coefficient	-.109	-.041
			Sig. (2-tailed)	.739	.893
			N	8	8
		Muc7 Resting	Correlation Coefficient	-.436	.454
			Sig. (2-tailed)	.182	.140
			N	8	8
		pH resting saliva	Correlation Coefficient	.316	-.527
			Sig. (2-tailed)	.480	.207
			N	5	5
		Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	.109	.371
			Sig. (2-tailed)	.739	.228
			N	8	8
		Cystatin S in resting saliva	Correlation Coefficient	.218	.124
			Sig. (2-tailed)	.505	.688
			N	8	8
	60+ Years Old	PrecriptionMed	Correlation Coefficient	1.000	.146
			Sig. (2-tailed)	.	.602
			N	11	11
		OTCMed	Correlation Coefficient	.146	1.000

		Sig. (2-tailed)	.602	.
		N	11	11
	Total Protein Resting Saliva	Correlation Coefficient	.181	-.106
		Sig. (2-tailed)	.465	.687
		N	11	11
	MUC5B Resting	Correlation Coefficient	-.221	-.317
		Sig. (2-tailed)	.372	.227
		N	11	11
	Muc7 Resting	Correlation Coefficient	.101	-.159
		Sig. (2-tailed)	.685	.546
		N	11	11
	pH resting saliva	Correlation Coefficient	-.265	.066
		Sig. (2-tailed)	.427	.849
		N	7	7
	Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	-.181	-.370
		Sig. (2-tailed)	.465	.159
		N	11	11
	Cystatin S in resting saliva	Correlation Coefficient	-.261	.000
		Sig. (2-tailed)	.291	1.000
		N	11	11

**. Correlation is significant at the 0.01 level (2-tailed).

9.7 Appendix 7. Effect of gender on taste, TRP and odour sensitivity and salivary parameters

Table 9-8 Independent Students T-Test – Effect of gender on UWMS composition and chemical properties

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Total Protein Resting Saliva	Equal variances assumed	2.898	.094	-2.033	54	.047	-3.69844	1.81901	-7.34533	-.05154
	Equal variances not assumed			-1.578	10.837	.143	-3.69844	2.34303	-8.86490	1.46803
MUC5B Resting	Equal variances assumed	.406	.526	1.012	54	.316	.28284	.27945	-.27742	.84310
	Equal variances not assumed			1.336	19.881	.197	.28284	.21164	-.15881	.72449
Muc7 Resting	Equal variances assumed	.130	.720	.111	54	.912	.01713	.15426	-.29214	.32640
	Equal variances not assumed			.125	15.277	.902	.01713	.13666	-.27369	.30795
pH resting saliva	Equal variances assumed	.694	.414	1.820	22	.082	.42556	.23376	-.05924	.91035
	Equal variances not assumed			1.658	7.481	.138	.42556	.25665	-.17351	1.02462

Carbonic anhydrase 6 in resting saliva	Equal variances assumed	.862	.357	.502	54	.618	.36622	.73001	-1.09736	1.82979
	Equal variances not assumed			.888	44.095	.379	.36622	.41234	-.46476	1.19719
Cystatin S in resting saliva	Equal variances assumed	.870	.355	.455	54	.651	.74743	1.64145	-2.54348	4.03834
	Equal variances not assumed			.917	53.642	.363	.74743	.81478	-.88635	2.38122

Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
Total Protein Resting Saliva	Female	46	10.9856*	4.75596	.70123
	Male	10	14.6840*	7.06972	2.23564
MUC5B Resting	Female	46	1.3329	.84326	.12433
	Male	10	1.0501	.54161	.17127
Muc7 Resting	Female	46	.7339	.45406	.06695
	Male	10	.7168	.37674	.11914
pH resting saliva	Female	18	6.8756	.47323	.11154
	Male	6	6.4500	.56618	.23114
Carbonic anhydrase 6 in resting saliva	Female	46	1.3205	2.26632	.33415
	Male	10	.9543	.76400	.24160
Cystatin S in resting saliva	Female	46	2.1678	5.13594	.75725
	Male	10	1.4204	.95097	.30072

Table 9-9 Effect of gender on spinnbarkeit of UWMS and SWMS

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
						Sig. (2-tailed)	Mean Difference	Std. Error Difference	95% Confidence Interval of the Difference	
		F	Sig.	t	df				Lower	Upper
Spinnbarkeit resting	Equal variances assumed	8.021	.006	1.991	54	.052	10.52242	5.28613	-.07564	21.12048
	Equal variances not assumed			3.719	50.592	.001	10.52242	2.82921	4.84143	16.20341
Spinnbarkeit water rinse	Equal variances assumed	.102	.751	.376	54	.708	2.07587	5.52095	-8.99296	13.14470
	Equal variances not assumed			.376	13.231	.713	2.07587	5.51398	-9.81528	13.96702
Spinnbarkeit menthol odour	Equal variances assumed	.009	.926	.678	54	.501	3.32934	4.91036	-6.51533	13.17402
	Equal variances not assumed			.617	12.105	.549	3.32934	5.39657	-8.41746	15.07614
Spinnbarkeit menthol	Equal variances assumed	4.186	.046	1.187	54	.240	5.55277	4.67691	-3.82388	14.92942
	Equal variances not assumed			2.386	53.732	.021	5.55277	2.32682	.88724	10.21830

Spinnbarkeit caffeine	Equal variances assumed	5.620	.021	1.294	54	.201	4.47299	3.45551	-2.45489	11.40086
	Equal variances not assumed			2.474	52.618	.017	4.47299	1.80793	.84613	8.09984
Spinnbarkeit MSG	Equal variances assumed	.302	.585	.208	54	.836	1.10441	5.30263	-9.52673	11.73554
	Equal variances not assumed			.232	15.025	.819	1.10441	4.75542	-9.03008	11.23890
Spinnbarkeit Capsaicin	Equal variances assumed	1.726	.194	.813	54	.420	7.58948	9.34049	-11.13708	26.31603
	Equal variances not assumed			.939	15.792	.362	7.58948	8.08379	-9.56578	24.74474

Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
Spinnbarkeit resting	Female	46	17.1561*	16.46886	2.42820
	Male	10	6.6337*	4.59159	1.45199
Spinnbarkeit water rinse	Female	46	11.4759	15.82843	2.33377
	Male	10	9.4000	15.79794	4.99575
Spinnbarkeit menthol odour	Female	46	16.1947	13.69603	2.01937
	Male	10	12.8653	15.82565	5.00451
Spinnbarkeit menthol	Female	46	10.3871*	14.63183	2.15735
	Male	10	4.8343*	2.75673	.87175
Spinnbarkeit caffeine	Female	46	8.7857*	10.78031	1.58947
	Male	10	4.3127*	2.72431	.86150
Spinnbarkeit MSG	Female	46	12.0917	15.57165	2.29592
	Male	10	10.9873	13.16922	4.16447
Spinnbarkeit Capsaicin	Female	46	23.9635	27.61229	4.07121
	Male	10	16.3740	22.08459	6.98376

Table 9-10 Effect of gender on UWMS and SWMS flow rate

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Resting flow rate	Equal variances assumed	.792	.377	.375	54	.709	.06097	.16272	-.26526	.38720
	Equal variances not assumed			.514	21.550	.613	.06097	.11863	-.18536	.30730
Water rinse flow rate	Equal variances assumed	5.794	.020	.896	54	.374	.32054	.35790	-.39701	1.03808
	Equal variances not assumed			1.735	53.449	.088	.32054	.18472	-.04990	.69097
Menthol odour flow rate	Equal variances assumed	.118	.733	-.161	54	.872	-.03753	.23253	-.50373	.42867
	Equal variances not assumed			-.209	19.058	.837	-.03753	.17993	-.41405	.33899
Menthol flow rate	Equal variances assumed	3.547	.065	.825	54	.413	.32465	.39356	-.46440	1.11370
	Equal variances not assumed			1.404	39.103	.168	.32465	.23127	-.14310	.79240
Caffeine flow rate	Equal variances assumed	2.868	.096	.549	54	.585	.21990	.40032	-.58270	1.02250

	Equal variances not assumed			.859	30.221	.397	.21990	.25591	-.30258	.74238
MSG flow rate	Equal variances assumed	3.194	.080	.789	54	.434	.45799	.58082	-.70648	1.62245
	Equal variances not assumed			1.300	35.411	.202	.45799	.35234	-.25701	1.17298
Capsaicin flow rate	Equal variances assumed	.919	.342	.459	54	.648	.30772	.67070	-1.03694	1.65239
	Equal variances not assumed			.504	14.733	.622	.30772	.61051	-.99561	1.61106

Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
Resting flow rate	Female	46	.7196	.49338	.07274
	Male	10	.6587	.29634	.09371
Water rinse flow rate	Female	46	1.0042	1.11744	.16476
	Male	10	.6837	.26416	.08354
Menthol odour flow rate	Female	46	.8138	.69965	.10316
	Male	10	.8513	.46619	.14742
Menthol flow rate	Female	46	1.1357	1.21834	.17963
	Male	10	.8110	.46063	.14566
Caffeine flow rate	Female	46	1.1062	1.23068	.18145
	Male	10	.8863	.57065	.18046
MSG flow rate	Female	46	1.4690	1.79356	.26445
	Male	10	1.0110	.73629	.23283
Capsaicin flow rate	Female	46	1.6441	1.96375	.28954
	Male	10	1.3363	1.69969	.53749

Table 9-11 Effect of gender on taste, TRP and odour perceptions

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Taste perception water rinse 1-10	Equal variances assumed	.206	.651	.322	54	.748	.1435	.4449	-.7486	1.0355
	Equal variances not assumed			.359	15.001	.724	.1435	.3995	-.7080	.9950
Taste perception menthol odour 1-10	Equal variances assumed	.659	.420	.508	54	.614	.3239	.6377	-.9546	1.6024
	Equal variances not assumed			.582	15.619	.569	.3239	.5561	-.8573	1.5052
Taste perception menthol 1-10	Equal variances assumed	5.434	.024	1.515	54	.136	.8152	.5381	-.2636	1.8940
	Equal variances not assumed			2.571	38.747	.014	.8152	.3171	.1736	1.4568
Taste perception caffeine 1-10	Equal variances assumed	.308	.581	.610	54	.544	.4609	.7552	-1.0532	1.9749
	Equal variances not assumed			.689	15.281	.501	.4609	.6689	-.9626	1.8843
Taste perception MSG 1-10	Equal variances assumed	.000	.993	-.176	54	.861	-.1239	.7036	-1.5345	1.2867
	Equal variances not assumed			-.183	13.784	.857	-.1239	.6767	-1.5773	1.3295

Taste perception capsaicin 1-10	Equal variances assumed	4.445	.040	3.021	54	.004	1.8478	.6116	.6216	3.0741
	Equal variances not assumed			2.238	10.559	.048	1.8478	.8258	.0209	3.6747

Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
Taste perception water rinse 1-10	Female	46	1.293	1.3063	.1926
	Male	10	1.150	1.1068	.3500
Taste perception menthol odour 1-10	Female	46	5.924	1.8826	.2776
	Male	10	5.600	1.5239	.4819
Taste perception menthol 1-10	Female	46	6.565*	1.6654	.2455
	Male	10	5.750*	.6346	.2007
Taste perception caffeine 1-10	Female	46	4.761	2.2230	.3278
	Male	10	4.300	1.8439	.5831
Taste perception MSG 1-10	Female	46	5.076	2.0357	.3001
	Male	10	5.200	1.9178	.6064
Taste perception capsaicin 1-10	Female	46	8.098*	1.5586	.2298
	Male	10	6.250*	2.5083	.7932

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
is taste sensitivity low or high	Equal variances assumed	75.546	.000	2.264	54	.028	.37826	.16705	.04334	.71318
	Equal variances not assumed			3.034	20.488	.006	.37826	.12468	.11858	.63794

Group Statistics

	Gender	N	Mean	Std. Deviation	Std. Error Mean
is taste sensitivity low or high	Female	46	1.4783*	.50505	.07447
	Male	10	1.1000*	.31623	.10000

9.8 Appendix 8. Correlation between PTC perception and taste, TRP and odour perceptions/responses

Table 9-12 Kendall's tau b correlation between PTC perception and taste, TRP and odour perceptions

Correlations

				PTC response 1-10
	Age of Participant			
Kendall's tau_b	18-30 Years Old	PTC response 1-10	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	20
		Taste perception water rinse 1-10	Correlation Coefficient	.327
			Sig. (2-tailed)	.062
			N	20
		Taste perception menthol odour 1-10	Correlation Coefficient	.266
			Sig. (2-tailed)	.135
			N	20
		Taste perception menthol 1-10	Correlation Coefficient	.285
			Sig. (2-tailed)	.103
			N	20
		Taste perception caffeine 1-10	Correlation Coefficient	.338
			Sig. (2-tailed)	.050
			N	20
		Taste perception MSG 1-10	Correlation Coefficient	.536**
			Sig. (2-tailed)	.002
			N	20
		Taste perception capsaicin 1-10	Correlation Coefficient	.196
			Sig. (2-tailed)	.273
			N	20
	60+ Years Old	PTC response 1-10	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	9

Taste perception water rinse 1-10	Correlation Coefficient	-.480
	Sig. (2-tailed)	.101
	N	9
Taste perception menthol odour 1-10	Correlation Coefficient	-.388
	Sig. (2-tailed)	.162
	N	9
Taste perception menthol 1-10	Correlation Coefficient	-.269
	Sig. (2-tailed)	.335
	N	9
Taste perception caffeine 1-10	Correlation Coefficient	-.435
	Sig. (2-tailed)	.112
	N	9
Taste perception MSG 1-10	Correlation Coefficient	.057
	Sig. (2-tailed)	.833
	N	9
Taste perception capsaicin 1-10	Correlation Coefficient	.000
	Sig. (2-tailed)	1.000
	N	9

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Table 9-13 Kendall's tau b correlation between PTC perception and SWMS response to taste, TRP and odour stimulation

Correlations

				PTC response 1-10
	Age of Participant			
Kendall's tau_b	18-30 Years Old	PTC response 1-10	Correlation Coefficient	1.000
			Sig. (2-tailed)	.
			N	20
		Resting flow rate	Correlation Coefficient	.312
			Sig. (2-tailed)	.065
			N	20
		Water rinse flow rate	Correlation Coefficient	.374*
			Sig. (2-tailed)	.027
			N	20
		Menthol odour flow rate	Correlation Coefficient	.223
			Sig. (2-tailed)	.187
			N	20
		Menthol flow rate	Correlation Coefficient	.329
			Sig. (2-tailed)	.052
			N	20
		Caffeine flow rate	Correlation Coefficient	.345*
			Sig. (2-tailed)	.041
			N	20
		MSG flow rate	Correlation Coefficient	.378*
			Sig. (2-tailed)	.025
			N	20
		Capsaicin flow rate	Correlation Coefficient	.323
			Sig. (2-tailed)	.056
			N	20
	60+ Years Old	PTC response 1-10	Correlation Coefficient	1.000
			Sig. (2-tailed)	.

	N	9
Resting flow rate	Correlation Coefficient	.229
	Sig. (2-tailed)	.399
	N	9
Water rinse flow rate	Correlation Coefficient	.171
	Sig. (2-tailed)	.527
	N	9
Menthol odour flow rate	Correlation Coefficient	-.114
	Sig. (2-tailed)	.673
	N	9
Menthol flow rate	Correlation Coefficient	.000
	Sig. (2-tailed)	1.000
	N	9
Caffeine flow rate	Correlation Coefficient	-.057
	Sig. (2-tailed)	.833
	N	9
MSG flow rate	Correlation Coefficient	.114
	Sig. (2-tailed)	.673
	N	9
Capsaicin flow rate	Correlation Coefficient	-.114
	Sig. (2-tailed)	.673
	N	9

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

9.9 Appendix 9. pH of SWMS compared to UWMS

Table 9-14 Holm-Sidak's multiple comparisons of mean difference between pH of USWMS to SWMS in older adults

Holm-Sidak's multiple comparisons test	Mean Diff.	Significant?	Summary		A-?	
Resting vs. Water Rinse	-0.501	Yes	***		B	Water Rinse
Resting vs. Menthol Odour	-0.33	Yes	**		C	Menthol Odour
Resting vs. Menthol	-0.625	Yes	****		D	Menthol
Resting vs. Caffeine	-0.547	Yes	****		E	Caffeine
Resting vs. Capsaicin	-0.702	Yes	****		F	Capsaicin
Resting vs. MSG	-0.539	Yes	****		G	MSG
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2
Resting vs. Water Rinse	6.825	7.326	-0.501	0.1172	11	11
Resting vs. Menthol Odour	6.825	7.155	-0.33	0.1172	11	11
Resting vs. Menthol	6.825	7.45	-0.625	0.1172	11	11
Resting vs. Caffeine	6.825	7.372	-0.547	0.1172	11	11
Resting vs. Capsaicin	6.825	7.527	-0.702	0.1172	11	11
Resting vs. MSG	6.825	7.364	-0.539	0.1172	11	11

Table 9-15 Holm-Sidak's multiple comparisons of mean difference between pH of USWMS to SWMS in younger adults

Holm-Sidak's multiple comparisons test	Mean Diff.	Significant?	Summary		A-?	
Resting vs. Water Rinse	-0.3857	No	ns		B	Water Rinse
Resting vs. Menthol Odour	-0.4114	No	ns		C	Menthol Odour
Resting vs. Menthol	-0.4507	Yes	*		D	Menthol
Resting vs. Caffeine	-0.3407	No	ns		E	Caffeine
Resting vs. Capsaicin	-0.57	Yes	**		F	Capsaicin
Resting vs. MSG	-0.4786	Yes	*		G	MSG
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2
Resting vs. Water Rinse	6.729	7.115	-0.3857	0.1731	14	14
Resting vs. Menthol Odour	6.729	7.141	-0.4114	0.1731	14	14
Resting vs. Menthol	6.729	7.18	-0.4507	0.1731	14	14
Resting vs. Caffeine	6.729	7.07	-0.3407	0.1731	14	14
Resting vs. Capsaicin	6.729	7.299	-0.57	0.1731	14	14
Resting vs. MSG	6.729	7.208	-0.4786	0.1731	14	14

9.10 Appendix 10. Effect of viscoelasticity on dynamic viscosity measurements

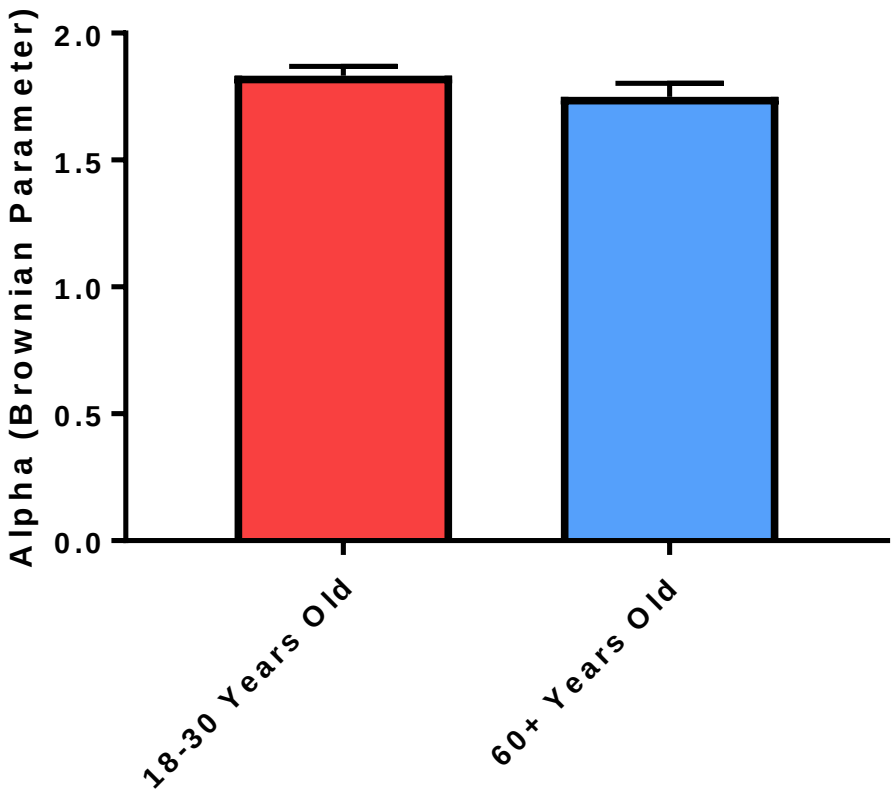


Figure 9-2 Alpha values (Brownian parameter) for dynamic viscosity of UWMS from older and younger adults. Measured using DDM. Coefficient alpha indicates how Brownian the samples are. Older (n=24) and younger (n=30). A value of 2 equates to a purely viscous fluid. A value below 2 indicates some viscoelasticity or drift of sloughed epithelial cells/mucin clusters in the sample. An alpha coefficient <1.5 indicates strong influence of drifting cell/mucin clusters on the measured dynamic viscosity. The calculation for Brownian parameter is $D = RT/NA6\pi\eta a = kBT/6\pi\eta a$ where R is the gas constant, NA = 6.06 × 1023/mol is Avogadro's number, T is the temperature, η is the viscosity of the liquid and a is the radius of the particle. kB = R/NA is Boltzmann's constant.

Table 9-16 Kendall's tau b correlation between UWMS spinnbarkeit and dynamic viscosity

Correlations

			Spinnbarkeit resting	Viscosity in UWMS
Kendall's tau_b	Spinnbarkeit resting	Correlation Coefficient	1.000	.003
		Sig. (2-tailed)	.	.976
		N	56	54
	Viscosity in UWMS	Correlation Coefficient	.003	1.000
		Sig. (2-tailed)	.976	.
		N	54	54

9.11 Appendix. 11 Correlation between UWMS physical properties and taste/TRP/olfactory responses

Table 9-17 Kendall's tau b correlation between UWMS spinnbarkeit and taste, TRP and odour perceptions

Correlations

			Spinnbarkeit resting
Kendall's tau_b	Spinnbarkeit resting	Correlation Coefficient	1.000
		Sig. (2-tailed)	.
		N	56
	PTC response 1-10	Correlation Coefficient	.340*
		Sig. (2-tailed)	.012
		N	29
	is taste sensitivity low or high	Correlation Coefficient	.219*
		Sig. (2-tailed)	.048
		N	56
	Taste perception water rinse 1-10	Correlation Coefficient	.095
		Sig. (2-tailed)	.336
		N	56
	Taste perception menthol odour 1-10	Correlation Coefficient	.059
		Sig. (2-tailed)	.539
		N	56
	Taste perception menthol 1-10	Correlation Coefficient	.014
		Sig. (2-tailed)	.886
		N	56
	Taste perception caffeine 1-10	Correlation Coefficient	-.052
		Sig. (2-tailed)	.584
		N	56
	Taste perception MSG 1-10	Correlation Coefficient	.213*
		Sig. (2-tailed)	.026
		N	56
	Taste perception capsaicin 1-10	Correlation Coefficient	.135
		Sig. (2-tailed)	.167
		N	56

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 9-18 Kendall's tau b correlation between UWMS dynamic viscosity and taste, TRP and odour perceptions

Correlations

			Viscosity in UWMS
Kendall's tau_b	Viscosity in UWMS	Correlation Coefficient	1.000
		Sig. (2-tailed)	.
		N	54
	Taste perception water rinse 1-10	Correlation Coefficient	.021
		Sig. (2-tailed)	.837
		N	54
	Taste perception menthol odour 1-10	Correlation Coefficient	-.065
		Sig. (2-tailed)	.506
		N	54
	Taste perception menthol 1-10	Correlation Coefficient	.064
		Sig. (2-tailed)	.512
		N	54
	Taste perception caffeine 1-10	Correlation Coefficient	.042
		Sig. (2-tailed)	.668
		N	54
	Taste perception MSG 1-10	Correlation Coefficient	.050
		Sig. (2-tailed)	.609
		N	54
	Taste perception capsaicin 1-10	Correlation Coefficient	-.017
		Sig. (2-tailed)	.861
		N	54
	PTC response 1-10	Correlation Coefficient	.138
		Sig. (2-tailed)	.318
		N	28
	is taste sensitivity low or high	Correlation Coefficient	-.017
		Sig. (2-tailed)	.882
		N	54

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

9.12 Appendix 12. Muco-Adhesion Assay Saliva Titration

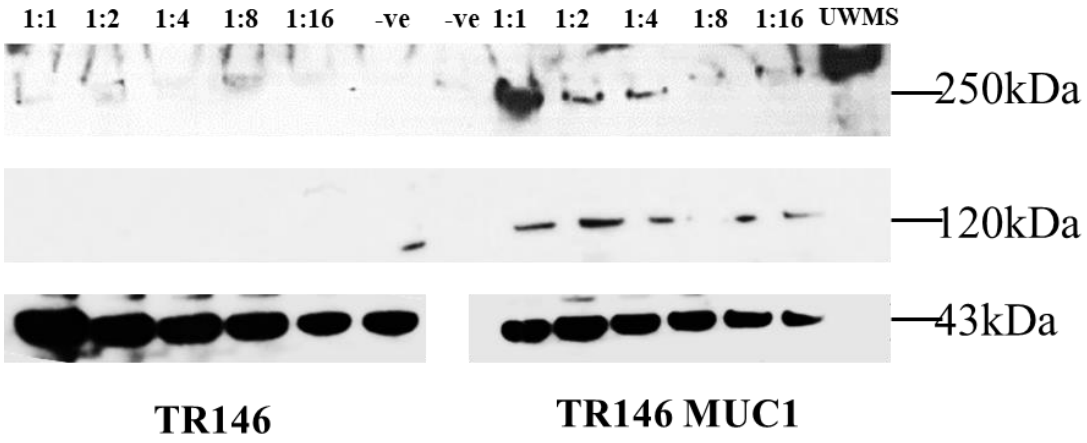


Figure 9-3 Titration of TR146 mucin binding from UWMS control (sample from one healthy donor). To determine saliva:media ratio required for muco-adhesion assay. Lanes 1-5 = TR146 cells, saliva diluted 1:1, 1:2, 1:4, 1:8 and 1:16 with media. Lane 6 = TR146 cells negative control (media only), Lane 7 = TR146 MUC1 cells negative control (media only). Lanes 8-12 = TR146/MUC1 cells, saliva diluted 1:1, 1:2, 1:4, 1:8 and 1:16 with media. Lane 13 = UWMS control sample run directly on gel. MUC5B = >250kDa, MUC1 = 120kDa, beta actin (used as loading control) = 43kDa.

9.13 Appendix 13. Confocal microscopy of calcium responses to PTC in TAS2R38 single and Gα16Gust44 double transfected TR146 cells

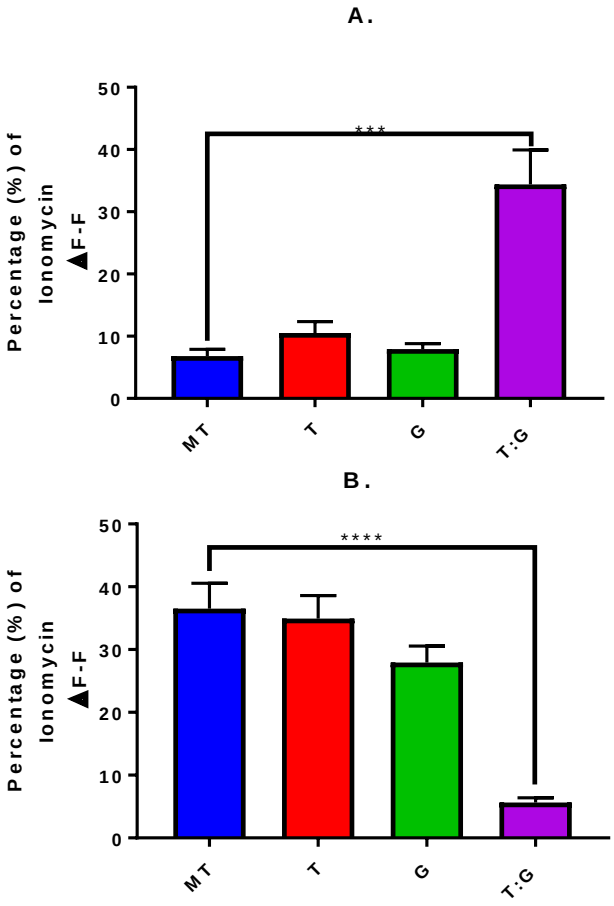


Figure 9-4 Confocal microscopy intracellular calcium measurements of singly and doubly TAS2R38:Gα16Gust44 transiently transfected TR146 cells with PTC. Cells were loaded with the fluo-4am calcium indicator and fluorescence emissions recorded before and after exposure of the cells to the bitter substance, PTC 50µm (A.) and 100µm (B.). A1R confocal microscopy was used to image cells at 40X magnification before and after compound addition. Fluorescence intensity, equating to calcium response, was quantified from the images using NIS Elements software (Nikon, UK). The panels show mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition, expressed as a ratio to change in fluorescence from baseline following ionomycin stimulation (maximum intensity) in TR146 cells, MT=mock-transfected, T=TAS2R38 transfected, G=Ga16Gust44 transfected, T:G=TAS2R38:Ga16Gust44 co-transfected TR146 cells. Data is representative of 2-3 experiments. Analysed for statistical significance using one-way ANOVA. Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****.

9.14 Appendix 14. Changes in mRNA in TR146 Cells Following PTC Stimulation

Table 9-19 Fold change in mRNA genes in TR146 cells before and after stimulation with 100μM PTC

control Avg (log2)	PTC Avg (log2)	Fold Change	Gene Symbol	Description
2.84	4.45	3.04	CLK2P1	CDC like kinase 2, pseudogene 1
2.21	3.73	2.87	APOBEC3D	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3D
2.48	4	2.86	KRTAP10-5	keratin associated protein 10-5
4.48	5.94	2.75	SNRPN; IPW	small nuclear ribonucleoprotein polypeptide N; imprinted in Prader-Willi syndrome (non-protein coding)
2.12	3.57	2.72	MIR4290 HG	MIR4290 host gene
2.62	4.06	2.71	IGKV1-9	immunoglobulin kappa variable 1-9
1.94	3.36	2.69	SNORD116-14	small nucleolar RNA, C/D box 116-14
2.42	3.82	2.64	SLC25A6	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
3.29	4.67	2.59	FAM86EP	family with sequence similarity 86, member A pseudogene
3.77	5.13	2.57	PRKX	protein kinase, X-linked
3.11	4.47	2.56	ZNF320	zinc finger protein 320
2.3	3.63	2.53	MIR924	microRNA 924
2.7	4.04	2.53	MIR378D2	microRNA 378d-2
3.68	5	2.51	LOC105372578	uncharacterized LOC105372578
1.54	2.86	2.49	GOLGA8DP	golgin A8 family, member D, pseudogene
2.28	3.59	2.48	RAB6C	RAB6C, member RAS oncogene family
2.24	3.55	2.48	LCA5	Leber congenital amaurosis 5
3.17	4.44	2.4	DTX2P1-UPK3BP1-PMS2P11	DTX2P1-UPK3BP1-PMS2P11 readthrough transcribed pseudogene
1.78	3.03	2.38	ACOT4	acyl-CoA thioesterase 4
1.91	3.16	2.38	KRTAP4-8	keratin associated protein 4-8
4.51	5.72	2.32	ACSL5	acyl-CoA synthetase long-chain family member 5
5.33	6.55	2.32	SNORD35B	small nucleolar RNA, C/D box 35B
3.39	4.6	2.32	MIR596	microRNA 596
3.34	4.54	2.3	SNORA80B	small nucleolar RNA, H/ACA box 80B
1.64	2.84	2.29	MIR548O2	microRNA 548o-2
2.71	3.87	2.25	FITM1	fat storage-inducing transmembrane protein 1
3.44	4.61	2.24	DDX11	DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 11
2.75	3.9	2.23	SNORD116-21	small nucleolar RNA, C/D box 116-21
2.71	3.87	2.23	ITFG1	integrin alpha FG-GAP repeat containing 1
2.41	3.56	2.21	GOLGA6L2	golgin A6 family-like 2
3.25	4.4	2.21	MIR4530	microRNA 4530

2.31	3.45	2.21	MIR3925	microRNA 3925
1.71	2.85	2.2	SNORD66	small nucleolar RNA, C/D box 66
1.69	2.83	2.2	MIR4798	microRNA 4798
1.44	2.57	2.19	MIR4438	microRNA 4438
2.93	4.05	2.17	IGLC7	immunoglobulin lambda constant 7
2.82	3.92	2.16	CPOX	coproporphyrinogen oxidase
3.41	4.52	2.15	P2RY8	purinergic receptor P2Y, G-protein coupled, 8
3.26	4.35	2.14	OR2L3	olfactory receptor, family 2, subfamily L, member 3
3.3	4.38	2.13	SNRPN	small nuclear ribonucleoprotein polypeptide N
2.21	3.3	2.13	SUGCT	succinyl-CoA:glutarate-CoA transferase
2.74	3.81	2.1	APOC2; APOC4; APOC4-APOC2	apolipoprotein C-II; apolipoprotein C-IV; APOC4-APOC2 readthrough (NMD candidate)
2.48	3.55	2.1	LOC105373363	uncharacterized LOC105373363
3.41	4.47	2.09	LOC646652	integral membrane glycoprotein-like
3.32	4.38	2.09	MIR339	microRNA 339
2.47	3.53	2.08	LINC00969	long intergenic non-protein coding RNA 969
2.34	3.39	2.07	LOC100129476	uncharacterized LOC100129476
3.56	4.59	2.05	C10orf25	chromosome 10 open reading frame 25
2.84	3.87	2.05	LOC105370623	uncharacterized LOC105370623
2.55	3.58	2.05	ZFP30	ZFP30 zinc finger protein
2.14	3.17	2.05	LOC101928505	uncharacterized LOC101928505
2.8	3.83	2.05	MIR3147	microRNA 3147
2.23	3.26	2.05	GPR20	G protein-coupled receptor 20
3.48	4.51	2.04	MIR606	microRNA 606
4.22	5.25	2.04	MCAT	malonyl-CoA-acyl carrier protein transacylase
1.77	2.8	2.04	MIR1302-7	microRNA 1302-7
1.92	2.95	2.03	LOC105376890	uncharacterized LOC105376890
2.69	3.72	2.03	YPEL2	yippee like 2
2	3.03	2.03	LOC401320	uncharacterized LOC401320
2.67	3.69	2.02	LOC105376116	uncharacterized LOC105376116
2.9	3.91	2.01	MIR3151	microRNA 3151
4.89	5.89	2	IPPK	inositol 1,3,4,5,6-pentakisphosphate 2-kinase
3.33	2.33	-2	LCE1C	late cornified envelope 1C
4.61	3.6	-2.01	SLC35G3	solute carrier family 35, member G3
3.08	2.07	-2.01	BAAT	bile acid-CoA:amino acid N-acyltransferase
3.31	2.3	-2.02	LOC105379516	uncharacterized LOC105379516
2.79	1.78	-2.02	ISX	intestine-specific homeobox
4.12	3.1	-2.02	NUPR2	nuclear protein 2, transcriptional regulator
4.9	3.88	-2.02	LOC441454	prothymosin, alpha pseudogene
2.98	1.96	-2.03	ELFN1	extracellular leucine-rich repeat and fibronectin type III domain containing 1

4.04	3.01	-2.04	CLEC11A	C-type lectin domain family 11, member A
3.04	2.01	-2.04	SNORD123	small nucleolar RNA, C/D box 123
3.33	2.3	-2.04	LOC105375095	uncharacterized LOC105375095
4.06	3.02	-2.05	RPL23AP64	ribosomal protein L23a pseudogene 64
5.11	4.07	-2.05	LOC105373890; LOC105373891	uncharacterized LOC105373890; uncharacterized LOC105373891
4	2.97	-2.05	PA2G4P4	proliferation-associated 2G4 pseudogene 4
3.27	2.24	-2.05	LOC401261	zinc finger protein ZIC 5
3.9	2.87	-2.05	LINC00997	long intergenic non-protein coding RNA 997
3.12	2.09	-2.05	LOC105375735	uncharacterized LOC105375735
3.08	2.04	-2.05	IFNA5	interferon, alpha 5
5.52	4.46	-2.08	LOC100130876	uncharacterized LOC100130876
4.89	3.84	-2.08	SCARNA27	small Cajal body-specific RNA 27
3.75	2.68	-2.09	AKR7A2	aldo-keto reductase family 7, member A2
2.97	1.91	-2.09	OR7C1	olfactory receptor, family 7, subfamily C, member 1
6.94	5.87	-2.09	SNORD13P2	small nucleolar RNA, C/D box 13 pseudogene 2
3.08	2	-2.1	C14orf178	chromosome 14 open reading frame 178
3.09	2.01	-2.11	MIR654	microRNA 654
4.33	3.25	-2.11	TRBV20OR9-2	T cell receptor beta variable 20/OR9-2 (non-functional)
2.99	1.9	-2.12	FCGR3A	Fc fragment of IgG, low affinity IIIa, receptor (CD16a)
3.47	2.39	-2.12	MAPT-IT1	MAPT intronic transcript 1
3.46	2.37	-2.13	ISM1	isthmin 1, angiogenesis inhibitor
3.36	2.27	-2.14	ZNF83	zinc finger protein 83
2.91	1.81	-2.15	PRKG1-AS1	PRKG1 antisense RNA 1
2.85	1.75	-2.15	MIR377	microRNA 377
3.87	2.76	-2.16	ALOX12-AS1	ALOX12 antisense RNA 1
3.65	2.54	-2.16	LINC01004	long intergenic non-protein coding RNA 1004
4.18	3.07	-2.16	SCARNA8	small Cajal body-specific RNA 8
3.97	2.85	-2.17	BRD1	bromodomain containing 1
5.73	4.6	-2.18	ZNF841; ZNF432	zinc finger protein 841; zinc finger protein 432
2.93	1.8	-2.19	DPF3	D4, zinc and double PHD fingers, family 3
5.34	4.2	-2.19	SMN1; SMN2	survival of motor neuron 1, telomeric; survival of motor neuron 2, centromeric
4.3	3.16	-2.2	SNORA75	small nucleolar RNA, H/ACA box 75
3.84	2.7	-2.2	IGHV1OR21-1	immunoglobulin heavy variable 1/OR21-1 (non-functional)
4.22	3.07	-2.21	MIR2117	microRNA 2117
4.44	3.28	-2.22	ASPN	asporin
6.71	5.55	-2.23	RPE65	retinal pigment epithelium-specific protein 65kDa
4.97	3.82	-2.23	SNORA71B; SNHG17	small nucleolar RNA, H/ACA box 71B; small nucleolar RNA host gene 17

5.33	4.16	-2.24	MIR1913	microRNA 1913
3.99	2.83	-2.24	FRMD8P1	FERM domain containing 8 pseudogene 1
3.13	1.96	-2.25	MTNR1B	melatonin receptor 1B
4.87	3.7	-2.25	PSG5	pregnancy specific beta-1-glycoprotein 5
5.33	4.16	-2.26	PRKXP1	protein kinase, X-linked, pseudogene 1
6.96	5.78	-2.26	NPIPB11	nuclear pore complex interacting protein family, member B11
4.7	3.51	-2.27	PMS2P9	PMS1 homolog 2, mismatch repair system component pseudogene 9
4.59	3.4	-2.28	LOC105373212	uncharacterized LOC105373212
4.12	2.93	-2.28	LOC102723496	uncharacterized LOC102723496
4.11	2.92	-2.29	MIR553	microRNA 553
4.15	2.95	-2.29	RUNX1-IT1	RUNX1 intronic transcript 1
2.85	1.63	-2.33	SNORD58C; RPL17	small nucleolar RNA, C/D box 58C; ribosomal protein L17
3.02	1.8	-2.33	CABP7	calcium binding protein 7
4.29	3.06	-2.34	LOC101927283; LOC105373558	uncharacterized LOC101927283; uncharacterized LOC105373558
5.84	4.6	-2.35	TRBV23-1	T cell receptor beta variable 23-1 (non-functional)
3.51	2.28	-2.35	MIR1206	microRNA 1206
4.99	3.75	-2.35	MIR4288	microRNA 4288
2.8	1.57	-2.35	SSX6	synovial sarcoma, X breakpoint 6 (pseudogene)
3.57	2.32	-2.38	TTLL7-IT1	TTLL7 intronic transcript 1
3.94	2.69	-2.38	SNORD23; GLTSCR2	small nucleolar RNA, C/D box 23; glioma tumor suppressor candidate region gene 2
5.07	3.81	-2.41	MMAB	methylmalonic aciduria (cobalamin deficiency) cblB type
6.32	5.05	-2.41	MIR221	microRNA 221
9.06	7.78	-2.43	MIR644A	microRNA 644a
5.02	3.72	-2.45	MIR494	microRNA 494
6.2	4.9	-2.47	PGAM4	phosphoglycerate mutase family member 4
3.59	2.29	-2.47	MIR766	microRNA 766
3.75	2.44	-2.48	MIR320C1	microRNA 320c-1
4.6	3.28	-2.49	PTEN	phosphatase and tensin homolog
4.2	2.88	-2.49	TRBV20OR9-2; TRBJ2-7	T cell receptor beta variable 20/OR9-2 (non-functional); T cell receptor beta joining 2-7
5.05	3.7	-2.54	MIR4689	microRNA 4689
4.45	3.09	-2.56	HPCAL1	hippocalcin-like 1
5.46	4.1	-2.57	SPIDR	scaffolding protein involved in DNA repair
5.43	4.05	-2.59	MIR4256	microRNA 4256
3.99	2.61	-2.61	MIR604	microRNA 604
5.35	3.97	-2.61	LOC100133299	GALI1870
4.09	2.69	-2.64	SCARNA11	small Cajal body-specific RNA 11
3.35	1.95	-2.65	IGKV1D-8	immunoglobulin kappa variable 1D-8
5.03	3.62	-2.66	SCARNA3	small Cajal body-specific RNA 3

6.44	5.02	-2.66	SLC25A3	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
3.37	1.93	-2.7	MIR4302	microRNA 4302
3.95	2.51	-2.7	MIR3137	microRNA 3137
3.3	1.85	-2.72	MIR548AL	microRNA 548al
5.47	4.01	-2.77	POTEH-AS1	POTEH antisense RNA 1
6.87	5.39	-2.78	NOTCH2NL	notch 2 N-terminal like
3.86	2.38	-2.79	IGLV1-50	immunoglobulin lambda variable 1-50 (non-functional)
4.04	2.56	-2.8	LOC105379520	uncharacterized LOC105379520
4.11	2.59	-2.86	PSG1	pregnancy specific beta-1-glycoprotein 1
4.09	2.58	-2.86	LOC100505658	uncharacterized LOC100505658
4.9	3.36	-2.9	TRBV23OR9-2	T cell receptor beta variable 23/OR9-2 (non-functional)
3.26	1.72	-2.91	MIR558	microRNA 558
3.59	2.05	-2.91	SNURFL	SNRPN upstream reading frame-like, pseudogene
4.39	2.83	-2.96	KCNRG; TRIM13	potassium channel regulator; tripartite motif containing 13
5.5	3.91	-3.01	SRGAP2; SRGAP2B	SLIT-ROBO Rho GTPase activating protein 2; SLIT-ROBO Rho GTPase activating protein 2B
5.52	3.92	-3.03	MFSD14C; ZNF782	major facilitator superfamily domain containing 14C; zinc finger protein 782
3.97	2.36	-3.04	GK3P	glycerol kinase 3 pseudogene
6.43	4.77	-3.14	SNORD92	small nucleolar RNA, C/D box 92
5.58	3.88	-3.26	KCNQ5-IT1	KCNQ5 intronic transcript 1
3.98	2.12	-3.63	FAM106CP	family with sequence similarity 106, member C, pseudogene
4.84	2.93	-3.75	MIR4742	microRNA 4742
4.1	2.16	-3.85	LOC105375699	uncharacterized LOC105375699
4.24	2.24	-3.98	LOC100303749	LSM3 homolog, U6 small nuclear RNA associated (S. cerevisiae) pseudogene
4.13	1.74	-5.27	YTHDC1	YTH domain containing 1
6.64	4.12	-5.72	SNORD56B	small nucleolar RNA, C/D box 56B

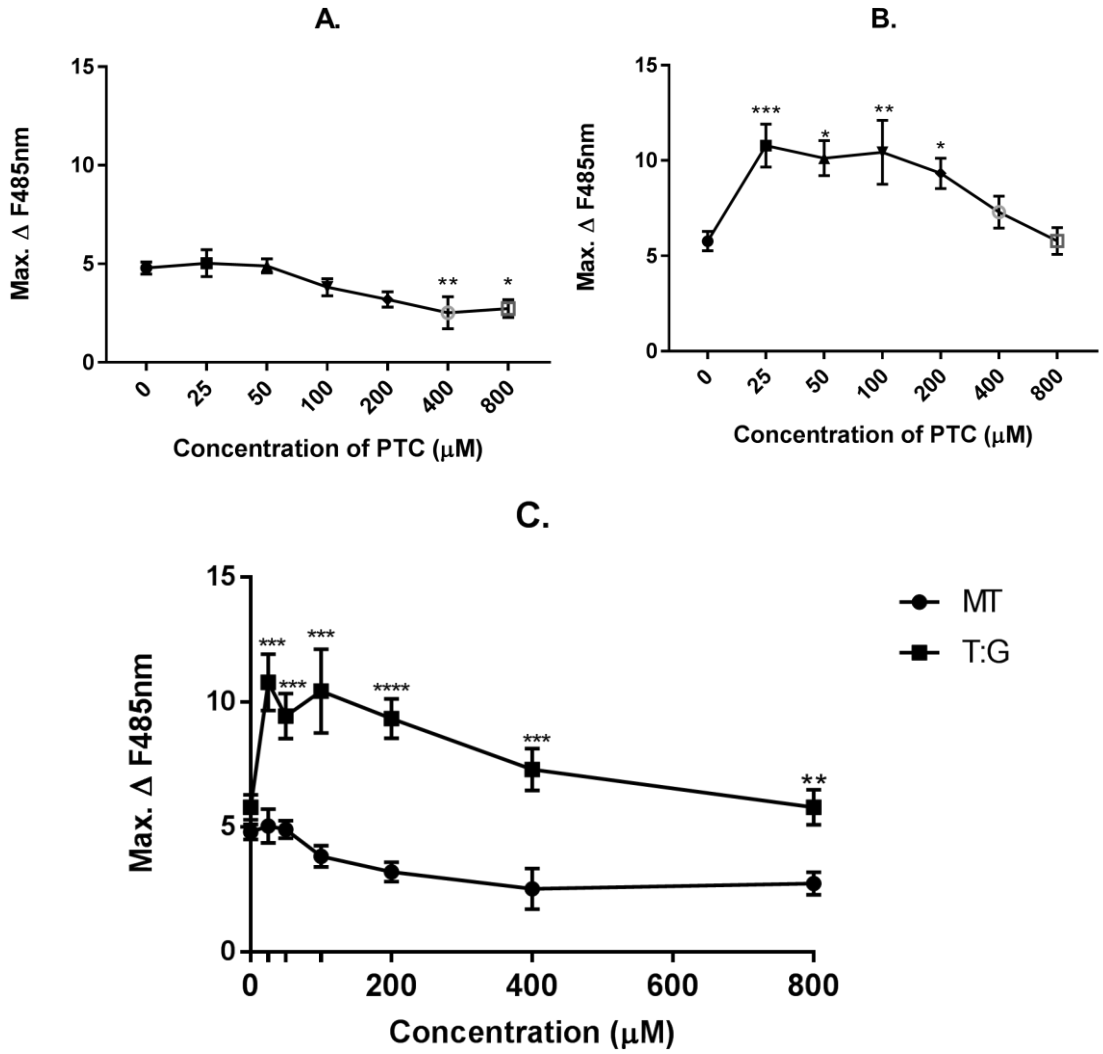


Figure 9-5 FLEX station intracellular calcium measurements of TR146 MUC1 cells with PTC FLUO4-am. Intracellular calcium changes in mock (A) and transfected TR146 MUC1 cells (B) loaded with the fluo-4am calcium indicator with increasing concentrations of PTC FLEX station fluorescence plate reader was used to measure intracellular calcium response (iCa^{2+}). The panels show mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition, in mock transfected (A.) and TAS2R38:Ga16Gust44 co-transfected (B.) TR146 MUC1 cells. Data is representative of 2-3 experiments (4-6 analyses). Analysed for statistical significance using one-way ANOVA with Tukey's multiple comparisons. Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****. Panel C. shows difference in responses between TAS2R38:Ga16Gust44 transfected and mock transfected cells. Analysed for statistical significance using students T test.

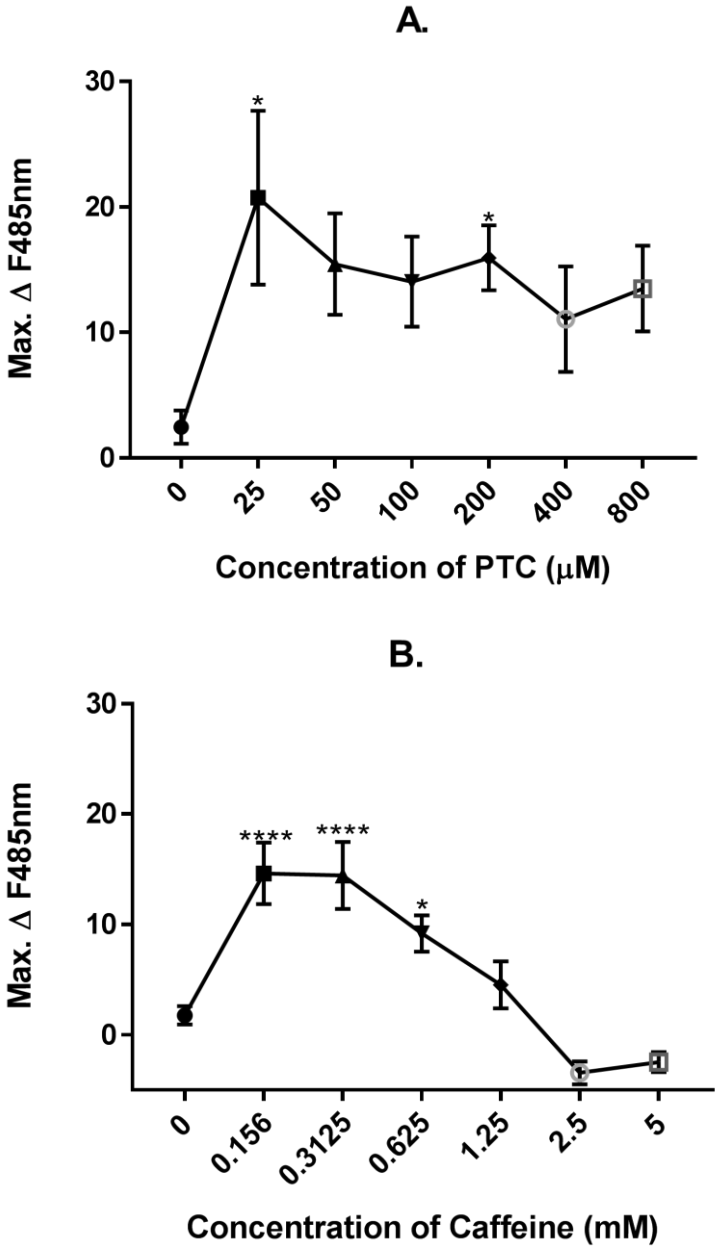


Figure 9-6 FLEX station intracellular calcium measurements of SCC090 cells with PTC and caffeine FLUO4-am. SCC090 cells were loaded with the fluo-4am calcium indicator and fluorescence emissions recorded before and after exposure of the cells to increasing concentrations of bitter receptor agonists the panels show mean (+/- SEM) change (Δ) in fluorescence from baseline after addition of compound, PTC (TAS2R38 agonist, A.) and caffeine (TAS2R10 agonist, B.). Data is representative of 2-3 experiments (4-6 analyses). Analysed for statistical significance using Kruskal-Wallis test with Dunn's multiple comparisons (A) or one-way ANOVA with Tukey's multiple comparisons test (B). Significance = P value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****.

9.15 Appendix 15. qPCR of Gα16Gust44 expression in transiently transfected TR146 cells

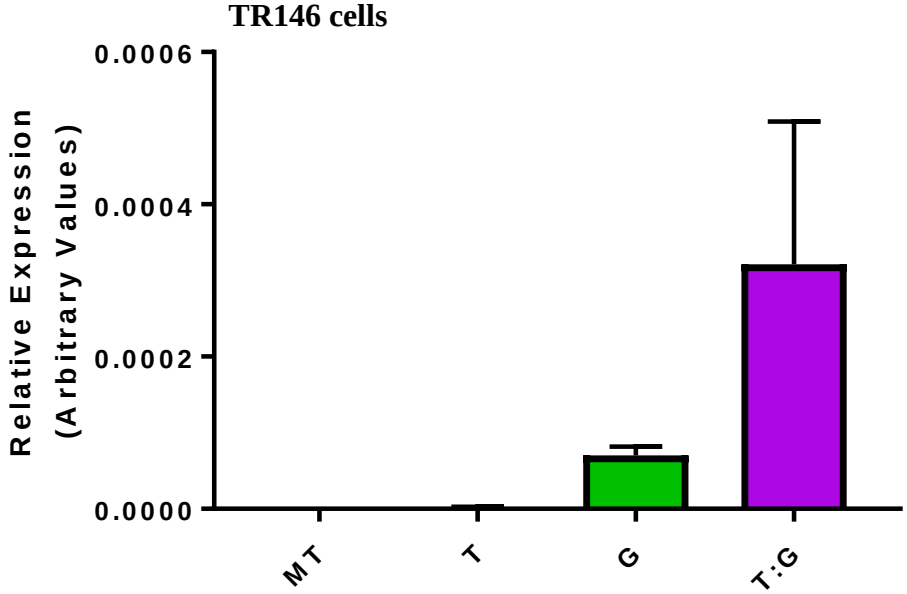


Figure 9-7 Quantification of Ga16Gust44 gene expression in TR146 cells, transiently transfected with cDNA for TAS2R38 and chimeric Ga16Gust44. Determined by qRT-PCR DeltaDeltaCT method. Mean relative expression of Ga16Gust44 (+/- SEM) in MT (mock transfected), T (TAS2R38 transfected), G (Ga16Gust44 transfected and T:G (TAS2R38:Ga16Gust44 co-transfected) TR146 cells. Data represent n = 2-3 (4-6 analyses).

9.16 Appendix 16. Expression of Sweet and Umami Taste Receptors in SCC090 and TR146 Cells

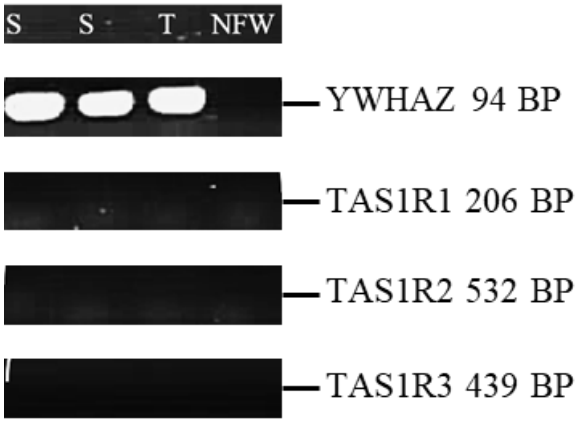


Figure 9-8 mRNA from SCC090 cells were used for RT-PCR of sweet and umami taste receptors. TR146 cell mRNA used as negative control. Representative image of agarose gel of PCR products using TAS1R1 (206Bp), TAS1R2 (532Bp) and TAS1R3 (439Bp) primers. YWHAZ housekeeping gene used as cDNA control (94Bp). S=SCC090 (duplicate samples), T=TR146, NFW=nuclease free water (negative control). Image representative of 2-3 biological replicates (4-6 analyses).

9.17 Appendix 17. Optimisation of TAS2R38:Gα16Gust44 Functional Expression in Transfected TR146 MUC1 Cells

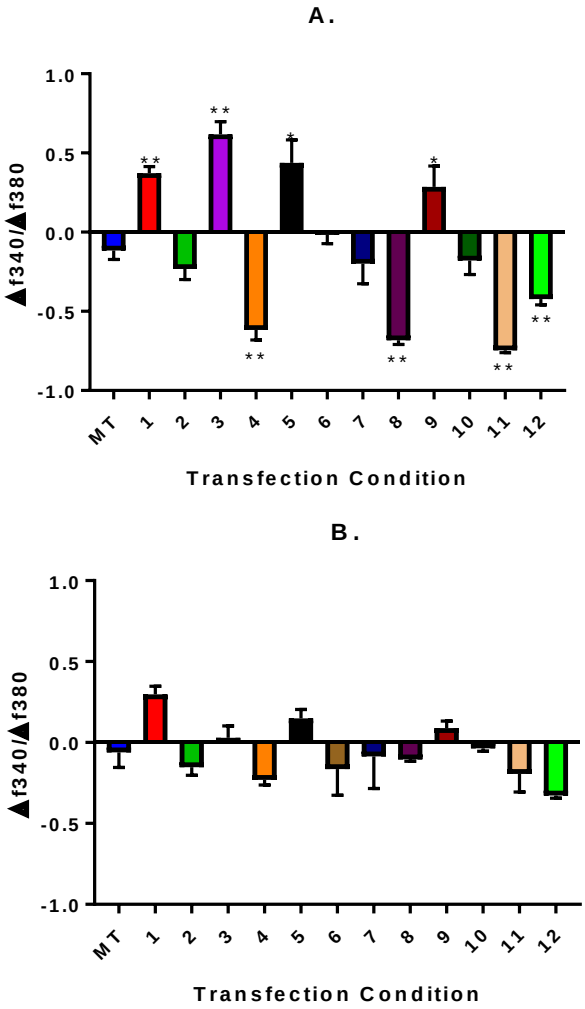


Figure 9-9 Transfection optimisation using FLEX station intracellular calcium measurements of PTC responses in transiently transfected TR146 MUC1 cells. TR146 MUC1 cells were transiently transfected with varying ratios of TAS2R38:Ga16Gust44 and transfection enzyme (1-12) or mock transfected using an empty vector as a negative control (MT). Cells were loaded with the fura2-am calcium indicator and intracellular calcium responses (fluorescence emissions) recorded before and after exposure of the cells to PTC (25µM) using FLEX station fluorescent plate reader. The panels show mean (+/- SEM) change (Δ) in fluorescence at 340nm after compound addition as a ratio to Δ fluorescence at 380nm (response to carrier control subtracted from all values). Transfection conducted 24 hours (A.) and 48 hours (B.) prior to assay. Data is representative of 1 experiments (3 analyses). Analysed for statistical significance using student's T-Test. Significance = P value < 0.05 * P < 0.01 **.

9.18 Appendix 18. Calcium responses to PROP in SCC090 and TR146 MUC1 cells

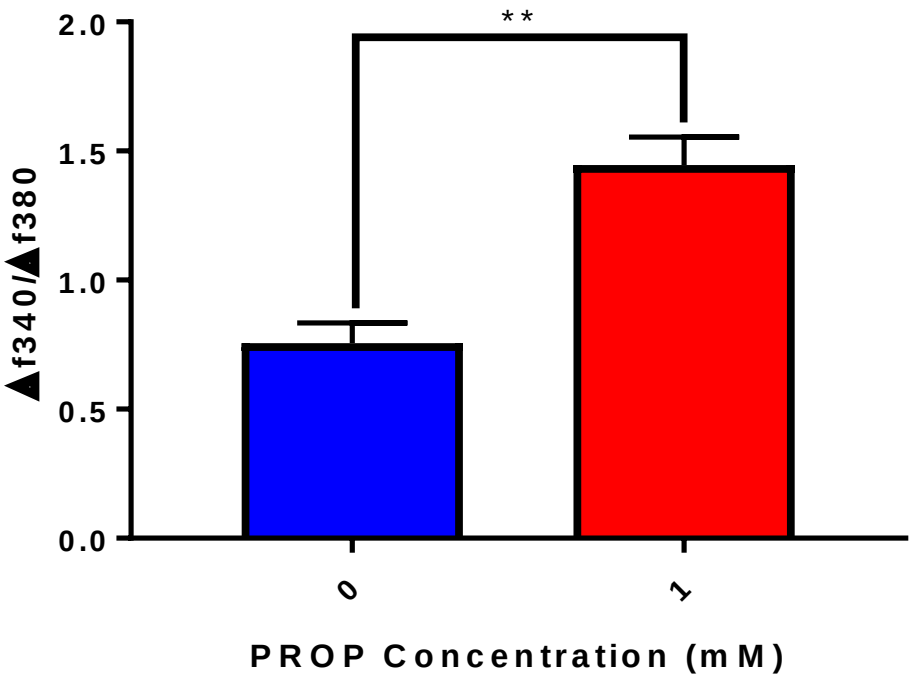


Figure 9-10 FLEX station measurement of intracellular calcium in SCC090 cells with PROP. SCC090 cells were loaded with the fura2-am calcium indicator and fluorescence emissions recorded before and after exposure of the cells to 1mM PROP. FLEX station fluorescent plate reader was used to measure intracellular calcium response (iCa^{2+}). The panels show mean (+/- SEM) change (Δ) in fluorescence at 340nm after compound or carrier control addition as a ratio to Δ fluorescence at 380nm. Data is representative of 2 experiments (6 analyses). Analysed for statistical significance using Mann-Whitney U Test. Significance = p value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****.

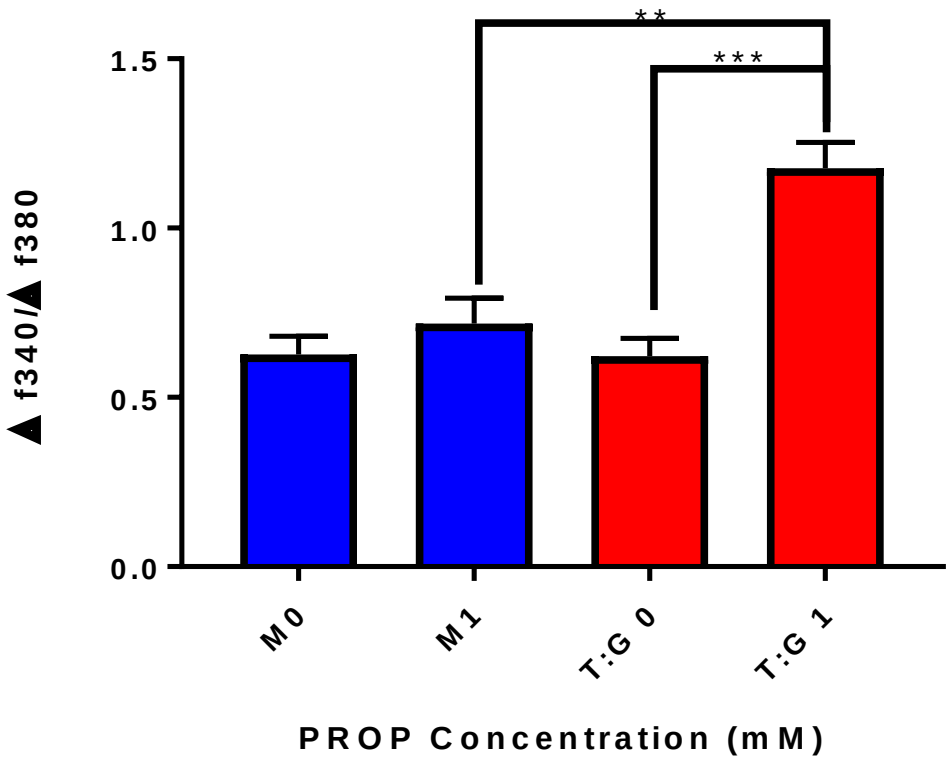


Figure 9-11 FLEX station measurement of intracellular calcium in TR146 MUC1 cells with PROP. TR146 MUC1 cells were loaded with the fura2-am calcium indicator and fluorescence emissions recorded before and after exposure of the cells to 1mM PROP. FLEX station fluorescent plate reader was used to measure intracellular calcium response (iCa^{2+}). The panels show mean (+/- SEM) change (Δ) in fluorescence from baseline after compound addition, in mock transfected (M) and TAS2R38:Ga16Gust44 co-transfected (T:G) TR146 MUC1 cells. Data is representative of 2 experiments (6 analyses). Analysed for statistical significance using student's T-Test. Significance = p value < 0.05 * P < 0.01 ** P < 0.001 ***, P<0.0001 ****.

9.19 Appendix 19. Confirmation of TAS2R38 and HSV tag primer efficiency

Table 9-20 Confirmation of primer efficiency using qPCR for TAS2R38 (A) and HSV tag (B) in plasmid DNA. Plasmid DNA was serially diluted and calculated concentration (ng/µl) determined using qPCR. Known concentrations of plasmid DNA (calculated by NanoDrop) and relative expression of YWHAZ housekeeping gene used to determine concentration of gene of interest. Data representative of 2 experiments.

A.	Ct (Mean +/-SD)	Given Concentration (ng/ul)	Mean Calculated Concentration (ng/ul)	Standard Deviation
STANDARD 1	5.31 +/- 0.14	183.8	139.87	17.05
STANDARD 2	5.84 +/- 0.02	84.8	89.98	1.93
STANDARD 3	6.64 +/- 0.26	39.6	46.62	9.84
STANDARD 4	7.51 +/- 0.02	19.4	22.28	0.32
STANDARD 5	8.28 +/- 0.03	8.4	11.71	0.30
STANDARD 6	10.24 +/- 1.17	3.4	2.83	2.40

B.	Ct (Mean +/-SD)	Given Concentration (ng/ul)	Mean Calculated Concentration (ng/ul)	Standard Deviation
STANDARD 1	6.31 +/- 0.14	183.8	183.77	52.54
STANDARD 2	7.22 +/- 0.02	84.8	80.85	11.76
STANDARD 3	7.25 +/- 0.26	39.6	78.77	12.15
STANDARD 4	9.60 +/- 0.02	19.4	24.25	31.34
STANDARD 5	10.08 +/- 0.03	8.4	15.03	19.20
STANDARD 6	10.43 +/- 1.17	3.4	5.77	4.62

9.20 Appendix 20 Correlation analysis of calcium responses to PTC and Caffeine in TR146 MUC1 and SCC090 cells with taste and salivary parameters

Table 9-21 Kendall's tau b correlation between calcium response to caffeine in SCC090 cells/PTC in TR146 cells and PTC perceptions in older and younger adults. Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of agonist, in presence of saliva from older and younger adults. Average perceived intensity of PTC by subjective measurement on a scale of 0-10 (data presented in chapter 3).

Correlations

		Response to caffeine in SCC090 cells	Response to PTC in TR146 MUC1
PTC response 1-10	Correlation Coefficient	.107	-.050
	Sig. (2-tailed)	.449	.719
	N	27	28

Table 9-22 Kendall's tau b correlation between calcium response to caffeine in SCC090 cells/PTC in TR146 cells and salivary protein levels, in older and younger adults. Calcium responses were recorded using FLEX station fluorescence plate reader, before and after addition of agonist, in presence of saliva from older and younger adults. Carbonic anhydrase 6 and cystatin S measured using immuno-blot of UWMS. Total protein quantified using BCA assay (data presented in chapter 3).

Correlations

				Response to caffeine in SCC090 cells	Response to PTC in TR146 MUC1
	Age of Participant				
Kendall's tau_b	18-30 Years Old	Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	-.034	-.099
			Sig. (2-tailed)	.789	.443
			N	30	30
		Cystatin S in resting saliva	Correlation Coefficient	.218	-.195
			Sig. (2-tailed)	.090	.129
			N	30	30
		Total Protein Resting Saliva	Correlation Coefficient	.025	-.131
			Sig. (2-tailed)	.844	.309
			N	30	30
	60+ Years Old	Carbonic anhydrase 6 in resting saliva	Correlation Coefficient	.209	.239
			Sig. (2-tailed)	.162	.102
			N	23	24
		Cystatin S in resting saliva	Correlation Coefficient	.391**	-.181
			Sig. (2-tailed)	.009	.215
			N	23	24
		Total Protein Resting Saliva	Correlation Coefficient	-.083	.246
			Sig. (2-tailed)	.579	.092
			N	23	24

Table 9-23 Kendall's tau b correlation between calcium response to caffeine in SCC090 cells/PTC in TR146 cells and rheology of UWMS, in older and younger adults. Calcium responses were recorded using FLEX station fluorescence plate reader,

before and after addition of agonist, in presence of saliva from older and younger adults. Viscoelasticity (spinnbarkeit) measured using NevaMeter. Viscosity measured using DDM (data presented in chapters 3 and 4).

Correlations

	Age of Participant			Response to PTC in TR146 normalised to control saliva	Response to PTC in TR146
Kendall's tau_b	18-30 Years Old	Spinnbarkeit resting	Correlation Coefficient	-.085	-.182
			Sig. (2-tailed)	.509	.159
			N	30	30
		Viscosity in UWMS	Correlation Coefficient	-.187	-.103
			Sig. (2-tailed)	.154	.431
			N	29	29
	60+ Years Old	Spinnbarkeit resting	Correlation Coefficient	-.029	.007
			Sig. (2-tailed)	.843	.960
			N	24	24
		Viscosity in UWMS	Correlation Coefficient	.094	.029
			Sig. (2-tailed)	.519	.843
			N	24	24

9.21 Appendix 21. Western blots: Amylase loading control, Carbonic Anhydrase VI, Cystatin S

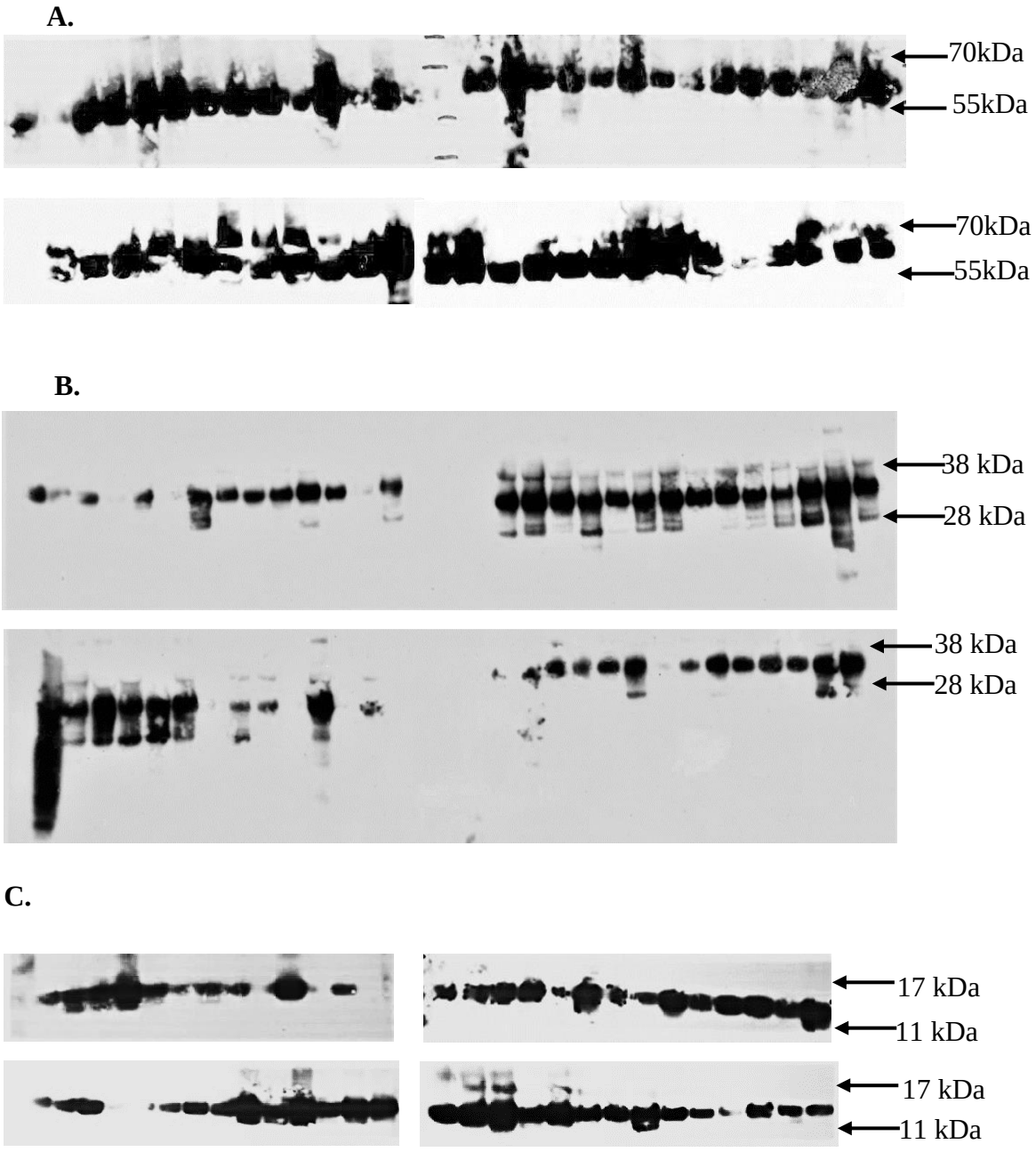


Figure 9-12 A. Human carbonic anhydrase VI, cystatin S and anti-amylase western blots of UWMS from older and younger adults. 18-30 years old N=31, 60+ years old N=25. A. anti-amylase western blots used as loading control for cystatin S and carbonic anhydrase VI blots. B. Carbonic anhydrase VI western blot images. C. Cystatin S western blot images.

Samples with no detectable amylase band were excluded from data analysis for carbonic anhydrase VI and cystatin S to give total numbers used for analysis: 18-30 N=28, 60+ N=23.

9.22 Appendix 22. Images of UWMS Lectin Blots

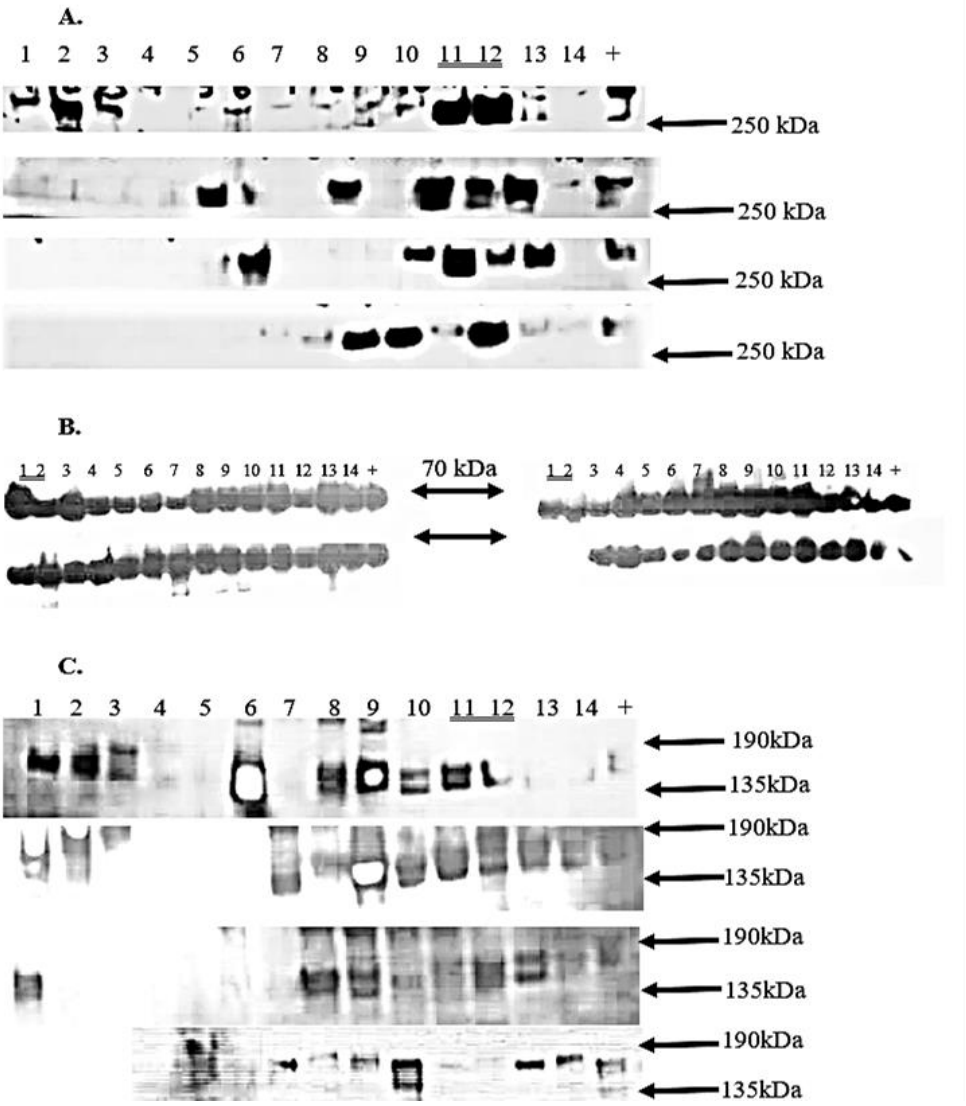


Figure 9-13 A. Images of SNA lectin immuno-blot in UWMS from older and younger adults. Sample order randomised to control for batch effects. Expected molecular weight >250kDa. B. Images of amylase immuno-blots (used as loading control) in UWMS from older and younger adults. Older (n=22) and younger (n=31) adults. Expected molecular weight 65kDa. C. Images of MAL II lectin immuno-blots in UWMS from older and younger adults. Expected molecular weight 155kDa. Lanes 1-14 = volunteer saliva samples, + = control UWMS sample from one healthy volunteer, used to normalise between gels.

9.23 Appendix 23. Images of mucin binding assay immuno-blots

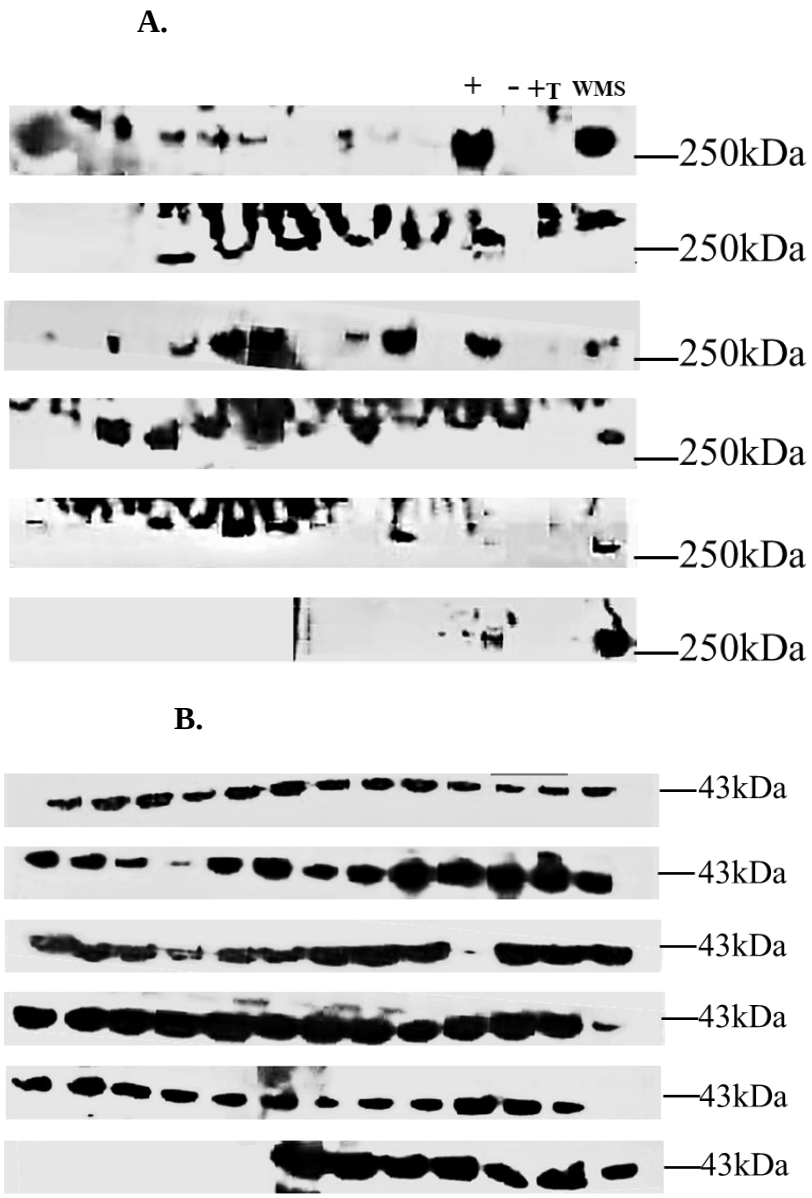


Figure 9-14 MUC5B from UWMS from older and younger adults binding to TR146/MUC1 cells A. Images of MUC5B immuno-blots. Sample order randomised to control for batch effects. Expected molecular weight >250kDa. B. Images of beta actin immuno-blots (used as loading control). Expected molecular weight 43kDa. Older (n=22) and younger (n=31). Lanes 1-10 = volunteer saliva sample binding, + = control UWMS sample from one healthy volunteer binding, - = negative control (media only), +T= Control UWMS binding to TR146 cells, WMS= WMS sample from one healthy donor, used to normalise between gels.

9.24 Appendix 24. Restriction Digest of Plasmid DNA to Confirm Purification of DNA

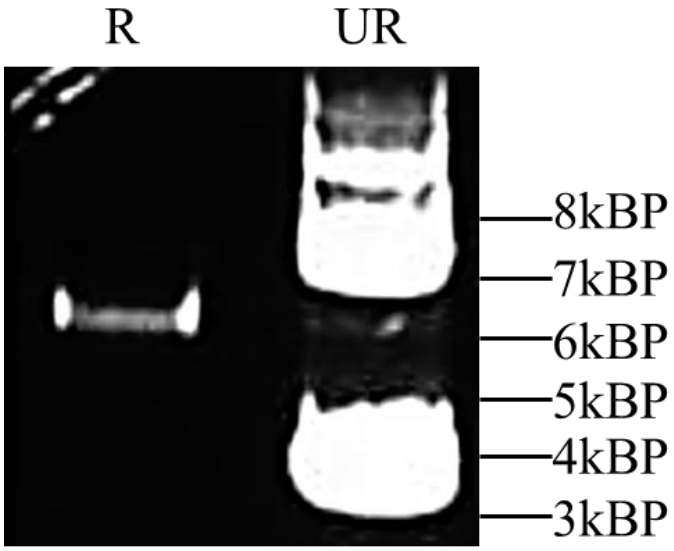


Figure 9-15 Agarose gel of NOTI restricted and un-restricted Ga16Gust44 plasmid DNA following transformation of XL-1 blue competent *E. coli* and maxi-prep purification. R=restricted, UR=un-restricted. Lane 1=NOTI restricted Ga16Gust44 plasmid DNA, lane 2=un-restricted Ga16Gust44 plasmid DNA. Predicted sizes: Vector=5400 base pairs (Bp), insert=1203Bp.

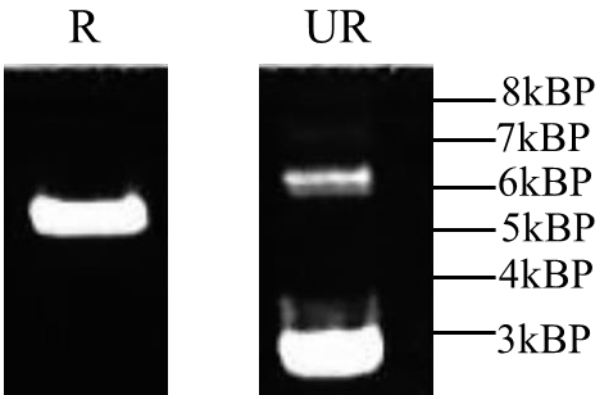


Figure 9-16 Agarose gel of ECORI restricted and un-restricted pcDNA3.1+ empty vector following transformation of XL-1 blue competent *E. coli* and maxi-prep purification. Lanes R=ECORI restricted pcDNA3.1+ empty vector, UR=un-restricted pcDNA3.1+ empty vector. Predicted sizes: Vector=5028 Bp. Samples run in duplicate.

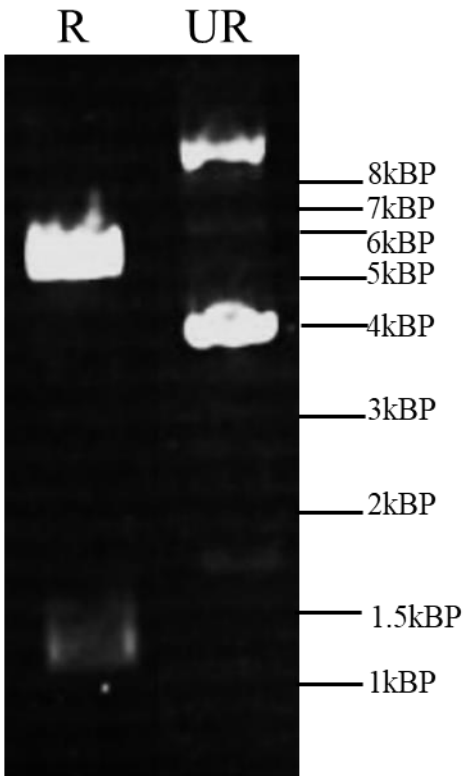


Figure 9-17 Agarose gel of HINDIII/NOTI double restricted and un-restricted TAS2R38 plasmid DNA following transformation of XL-1 blue competent *E. coli* and maxi-prep purification. R= HINDIII/NOTI double restricted TAS2R38 plasmid DNA, UR=un-restricted TAS2R38 plasmid DNA. Predicted sizes: Vector=4949 Bp, insert=1182Bp. Samples run in duplicate.

9.25 Appendix 25. Full nucleotide sequence of TAS2R38 plasmid DNA

pcDNA5FRT_sst3TAS2R38HSV

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGCTCTGATG
CCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTCGCTGAGTAGTGCGCGAGCAAAAT
TTAAGCTACAACAAGGCAAGGCTTGACCGACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCG
CTGCTTCGCGATGTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAA
TTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCC
TGGCTGACCGCCCAACGACCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTA
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GCCACCTGACGTC

Insert highlighted.

Unique Restriction sites: 916 KpnI GGTACC

2120 ApaI GGGCCC

1523 OliI CACNNNNGTG

9.26 Appendix. 26 Titration of UWMS to Optimise PTC Calcium Response Assay

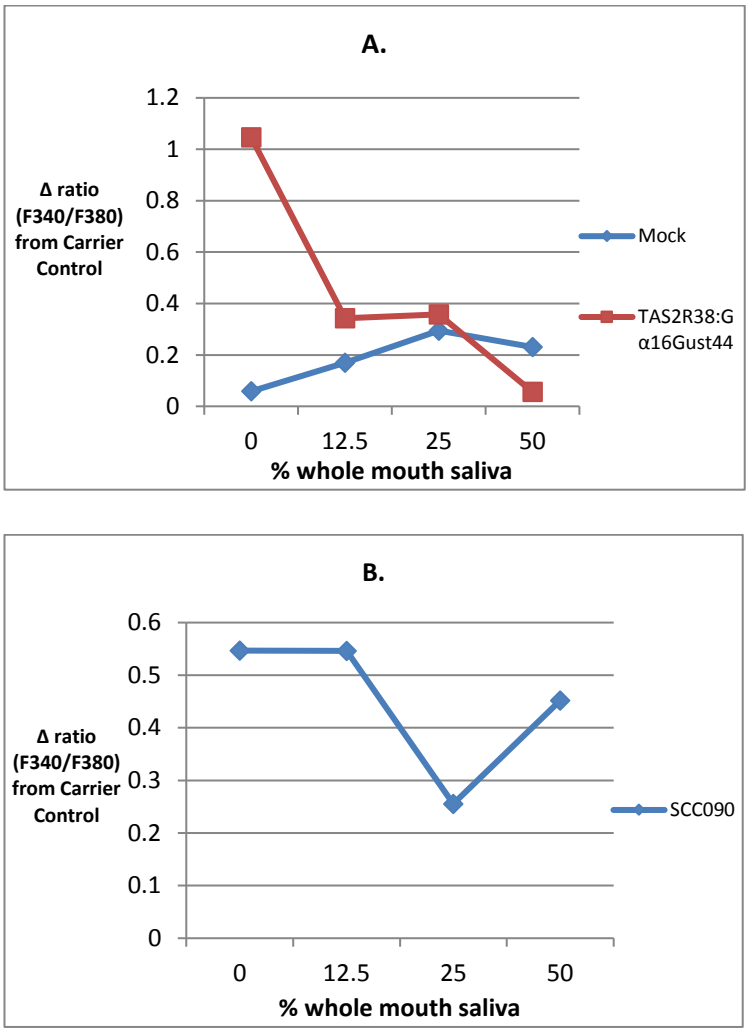

Figure 9-18 Titration of control WMS FLEX station intracellular calcium measurements of TR146 MUC1 and SCC090 cells with PTC.

TAS2R38:Gα16Gust44 and mock transfected TR146 MUC1 (A.) and SCC090 (B.) cells were loaded with the fura2-am calcium indicator and incubated with increasing concentrations of UWMS Fluorescence emissions recorded using FLEX station fluorescence plate reader, before and after exposure of the cells to PTC. The panels show mean (+/- SEM) peak response (fluorescence ratio 340nm:380nm) from baseline after addition of PTC. Data is representative of 1 preliminary experiment.

9.27 Appendix 27 Calcium Responses to Caffeine in TR146 MUC1 Cells

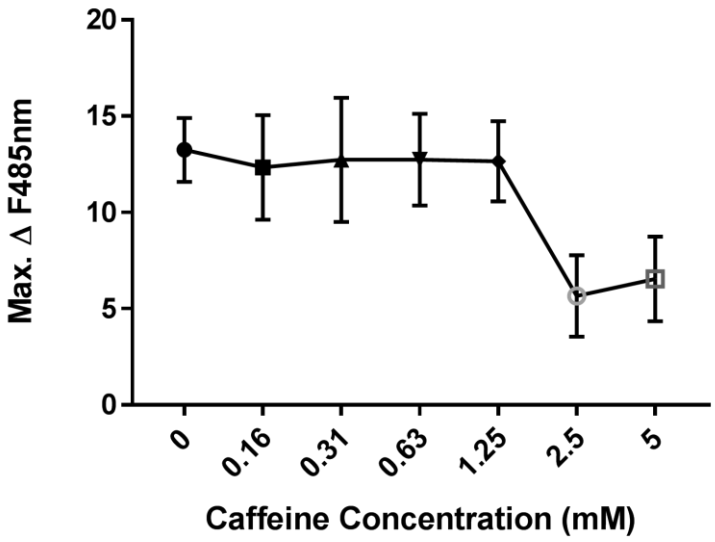

Figure 9-19 FLEX station intracellular calcium measurements of TR146 MUC1 cells with caffeine. TR146 MUC1 cells were loaded with the fluo-4am calcium indicator and incubated with or without UWMS. Fluorescence emissions were recorded using FLEX station fluorescence plate reader, before and after addition of increasing concentrations caffeine. The panels show mean (+/- SEM) change (Δ) in fluorescence from baseline addition of caffeine. Data is representative of 1 experiments (preliminary experiment, 4-6 analyses). Analysed for statistical significance using one-way ANOVA with Dunnetts's multiple comparisons.